MicroRNAs and Tissue Development

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Oslo, June 2011
Anne Marthe Jevnaker

Summary

Expression of microRNA (miRNA) in developing murine tissues was studied: focus being on the miR-17-92 cluster (oncomir-1) and two paralogous clusters, miR-106a-363 and miR-106b-25. Using microarrays we mapped the miRNA expression profile at selected times during development of two oral tissues: tooth germ and submandibular salivary gland. MiRNA expression in developing liver was also monitored. MiRNA expression was found to be highly abundant during development of these tissues: five of the miRNAs encoded in the miR-17-92 cluster exhibited decreased expression at postnatal stages compared to embryonic stages (Paper I).

Despite the oncogenic role of the miR-17-92 cluster, the function of the cluster in normal tissue remains unclear. However, studies have implicated the miR-17-92 cluster in development, and the cluster may be involved in regulation of cell cycle. The miR-106b-25 cluster has been associated with analogous functions.

Expression of the miR-17-92 primary transcript and all miRNAs encoded in this cluster was measured in nine different murine tissues at various developmental stages using real-time PCR. The relative levels of expression all miRNAs encoded in the miR-17-92 cluster exhibited decreased expression during development. Moreover, the pattern of expression of the primary transcript correlated to that of the mature miRNAs, although, the level of expression of the individual miRNAs were markedly different. These observations indicate that the level of expression of miRNAs encoded in the miR-17-92 cluster is regulated at a post-transcriptional level. To investigate possible involvement of members of the miR-17-92 cluster in proliferation the levels of expression of all members of the cluster were monitored at selected cell passages of cultured primary oral keratinocytes. The results indicate miRNAs of the miR-17-92 cluster may promote cell proliferation and inhibit differentiation (Paper II).

Possible functions of the members of the miR-106a-363 cluster, often considered as non-functional, was investigated by transfecting cells with miR-363* mimic. Transfection with miR-363* mimic led to decreased cellular proliferation and to decreased levels of expression of all miRNAs encoded by the miR-17-92 and miR-106b-25 clusters, suggesting that miR-363* may exert an inhibitory effect on expression of miRNAs derived from paralogous clusters. Therefore, the results suggest that the miRNAs*-species are not always by-products devoid of biological function (Paper III).

List of papers

This thesis is based on the following papers:

1) Jevnaker, A.M., and H. Osmundsen. 2008. MicroRNA expression profiling of the developing murine molar tooth germ and the developing murine submandibular salivary gland. *Arch Oral Biol*. 53:629-645.

2) Jevnaker, A.M., Khuu, C., Kjøle, E., Bryne, M. and H. Osmundsen. 2010. Expression of members of the miRNA17-92 cluster during development and in carcinogenesis. *Journal of Cellulare Physiology*. 226: 2257-2266.

3) Khuu, C., Jevnaker, A.M., Bryne, M. and H. Osmundsen. 2011. Regulation of expression of microRNAs encoded by paralogous, polycistronic, clusters. A possible role for miR-363*. Submitted to *Journal of Cellular Physiology*.

Abbreviations

3' UTR	3'-end untranslated region
5' UTR	5'-end untranslated region
5S	ribosomal 5S RNA
ADAR	adenosine deaminase, RNA-specific
AGO	argonaute
Amelx	amelogenin X chromosome
ANOVA	analysis of variance between groups
Bim	BCL2-like 11
C13orf25	chromosome 13 open reading frame 25
CDKN1A (p21)	cyclin-dependent kinase inhibitor 1A
cDNA	complementary deoxyribonucleic acid
Chr	chromosome
c-Myc	v-myc myelocytomatosis viral oncogene homolog
CpG	cytosine-phosphate-guanine
DGCR8	DiGeorge syndrome critical region gene 8
DNA	deoxyribonucleic acid
E	embryonal day
EC	endogene control
EMT	epithelial mesenchymal transitions
Enam	enamelin
ES cells	embryonic stem cells
GC	guanine-cytosine
HIF-1α	hypoxia inducible factor 1, alpha subunit
hnRNP A1	heterogeneous nuclear ribonucleoprotein A1
Kis2	kaplan integration site 2
LNA	locked nucleic acid
Mapk14	mitogen-activated protein kinase 14
MCM7	minichromosome maintenance complex component 7
MET	mesenchymal epithelial transition
MFE	minimum free energy
miR	microRNA
miRISC	miRNA-induced silencing complex
miRNA	microRNA
mRNA	messenger RNA
MVBs	multivesicular bodies
ncRNA	non-coding RNA
n-MYC	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
nt	nucleotides
P	postnatal day
PABPC	poly A binding protein, cytoplasmic 1

PAGE	polyacrylamide gel electrophoresis
P-bodies	processing bodies
PCR	polymerase chain reaction
pre-B cells	precursor B cell
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
pro-B cells	progenitor B cells
PTEN	phosphatase and tensin homolog
Ran-GTP	RAs-related Nuclear protein-guanosine-5'-triphosphate
RAS	RAt Sarcoma
Rbl2	retinoblastoma-like 2
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAPII	RNA polymerase II
Rnase III	ribonuclease type III
RNU48	small nucleolar RNA, C/D box 48
RT	reverse transcription
RT-PCR	real-time PCR
SGs	stress granules
shRNA	short hairpin RNA
SMG	submandibular salivary gland
snoRNA	small nucleolar RNA
STAT3	signal transducer and activator of transcription 3
TFIIB	transcription factor II B
TGFβ	transforming growth factor beta
Tm	melting temperature
TNFα	tumor necrosis factor alfa
TP53	tumor protein 53
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
tRNA	transfer RNA
U6	RNA, U6 small nuclear 1
ULS	universal linkage system

1 Introduction

1.1 What are microRNAs?

It has been assumed that biological processes are primarily controlled by regulatory proteins. However, the number of protein-coding genes in a genome is not correlated to the complexity of the organism. In fact, about 98% of transcriptional output in humans is non-protein-coding RNA [1]. These sequences have been considered to be evolutionary "garbage" without any function. During the last decade, however, a significant fraction of this non-coding RNA has been suggested to be highly relevant for the regulation of gene expression [2]. Strong association between the amount of non-coding sequences and biological complexity has also been found [1]. Therefore, an additional layer of regulatory RNA has been revealed (Fig. 1). Non-coding RNAs are suggested to act as a digital processing network, allowing increased regulatory complexity in biological systems, well beyond that achieved by protein-based analog regulatory networks [1]. One group of highly abundant and functionally important non-coding RNAs is microRNAs (miRNAs). MiRNAs are found in all eukaryotic cells [3].

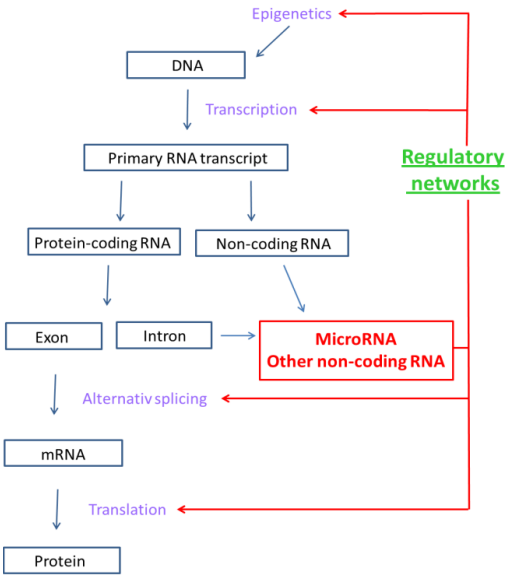


Fig.1. The flow of genetic information from DNA to protein is regulated by a network of interactions involving RNA molecules.

1.1.1 The discovery of microRNAs

MiRNAs were discovered in 1993 by Victor Ambros and colleagues during their study of the gene lin-14 in *C. elegans*. Ambros and his colleagues cloned the gene for lin-4, a repressor of lin-14. The lin-4 gene product turned out to be a short (~ 22-nucleotide), non-coding strand of RNA, containing sequences partially complementary to multiple sequences in the 3'UTR of the lin-14 mRNA. The researchers reported that this new "small antisense" lin-4 RNA was capable of down-regulating gene expression by directly binding the lin-14 mRNA [4]. Ambros et al. found that lin-4 regulated not only lin-14 but also lin-28, suggesting that miRNA functioned at multiple sites across the *C. elegans* genome [5].

Until the beginning of 2000 the discovery of the first miRNA was barely noticed by the scientific community. In 2000, Ruvken and colleagues discovered a second miRNA in *C. elegans*, let-7, whose sequence was shown to be evolutionarily conserved across the animal kingdom [6, 7]. These findings were the beginning of the small RNA revolution.

1.1.2 Biogenesis and basic functions of microRNAs

The biogenesis of miRNAs in animals was described by Bartel et al. in 2004 [8]. Transcription of miRNAs is regulated in a manner similar to that of protein-coding genes. About 40% of miRNA loci are located in the intronic region of non-protein coding transcripts and about 10% in the exonic region of non-protein coding sequences, ~40 % appear in the exonic region of protein-coding genes [9]. Transcription by polymerase II (or polymerase III) generates long primary transcripts (pri-miRNA) several kilo bases in length that are capped and polyadenylated (Fig.2) [8-10]. The primary transcripts may contain mono-cistronic or poly-cistronic hair-pin structures with ~32 nucleotides (nt) long imperfectly base-paired stems and terminal loops [11, 12]. The monocistronic transcripts encode individual miRNAs, while the poly-cistronic transcripts encode a number of coordinately expressed miRNAs, so called miRNA clusters [11, 13]. There are normally two or three miRNA genes in a cluster, however, larger cluster also exist [10].

The evolutionary conserved mechanism by which pri-miRNA are processed initially to precursor miRNAs (pre-miRNAs) and subsequently to mature miRNAs involves two endonucleolytic cleavages by the RNase III enzymes Drosha and Dicer [14, 15]. Drocha processes the pri-miRNA into a ~70 nt hairpin pre-miRNA. Subsequently, the pre-miRNA is transported into the cytoplasm through interaction with exportine-5 and Ran-GTP, where it

undergoes a second round of processing, this time catalysed by Dicer [15, 16]. The product is a ~22 nt miRNA:miRNA* duplex. Both Drocha and Dicer are assisted by a number of cofactors or accessory proteins, some of which have regulatory functions. The levels and activity of these proteins control the accumulation of miRNAs (e.g. DGCR8, TRBP) [14].

Epigenetic events, such as promoter methylation and histone acetylation, have also been shown to alter the miRNA expression. However, the interaction between microRNAs and the epigenetic machinery is complicated by the fact that microRNAs also target molecules involved in the methylation and acetylation of DNA and histones, therefore, also likely regulating these mechanisms [17].

One strand of the ~22 nt duplex (the miRNA or the miRNA*) is subsequently incorporated into the miRNA-induced silencing complex (miRISC) by associating with Argonaute (AGO) proteins [3, 14]. It was formerly assumed that the miRNA strand is selected for entry into miRISC, and that the miRNA* strand is degraded. However, recent studies has pointed out that the miRNA* species may have physiological roles [3].

The miRISC uses the retained miRNA as a guide strand to identify target mRNAs, and miRNAs regulate protein synthesis by base-pairing to target mRNAs [14]. The complementary canonical base-pairing between the "seed" sequence of the miRNA (nucleotides 2-8 at its 5'-end) and its complementary seed match sequence (which is present in the 3'-UTR of target mRNAs) determine the extent of inhibition of the target [3]. Perfect or near-perfect complementarity is required for mRNA cleavage; lower degrees of complementarity will lead to repression of protein synthesis. In animals, the latter mechanism is more common [3]. Endonucleolytic cleavage of the target mRNA is usually prevented by mismatches and bulges in the central region of the miRNA-mRNA duplexes [18].

The miRNA repress protein synthesis either by repressing translation and/or by bringing about deadenylation and subsequent mRNA decay [14, 19]. Several mechanisms have been proposed for the repression of protein synthsis by miRNAs, including inhibition at the level of initiation or elongation, as well as storage of mRNAs in P-bodies and SGs (stress granules) [14, 18]. Lately, multivesicular bodies (MVBs) and endosomes have also been recognized as cellular organelles that contribute to miRNA function [18]. The principal model is that translational inhibition leads to mRNA degradation in the absence of mechanism able to protect the transcript. However, the connection between translational inhibition and decay remains to be fully understood.

The “seed” sequence tends to be the most highly conserved region of a miRNA molecule [20]. Consequently, miRNAs are often grouped into families based on the “seed” sequence [21]. By means of the “seed” sequence, bioinformatic target prediction suggests that human miRNAs could potentially regulate up to 50% of the protein-coding genes in the human genome [14]. However, the significance of the “seed” sequence has been contradicted by studies showing the that sequences outside the “seed” are also crucial for gene regulation [22]. In animals, there is also growing evidence that binding of miRNAs within the 5’-UTR and coding regions of the target mRNA can lead to post-transcriptional control [23]. Additionally, a single miRNA molecule may have multiple effects because of its ability to regulate several targets [14]. On the other hand, several miRNAs can target the same mRNA, thereby modulating the effect of a single miRNA in a combinatorial manner.

Lately, a wide variety of mechanisms has been shown to generate miRNA-like molecules. Bypassing a key step involved in the canonical miRNA pathway, mitron, snoRNA, shRNA or tRNA have been suggested to yield subclasses of miRNAs. Consequently, non-canonical pathways of miRNA biogenesis presents an additional level of complexity to the miRNA-dependent regulation of mRNA expression (reviewed in [12]).

MiRNA may also have functions other than translational gene silencing. Several reports indicate that miRNAs act as activators of translation [14, 18]. In addition, mature mammalian miRNAs can be imported into the nucleus suggesting that miRNAs regulate transcription (Fig.2) [24]. Recent finding suggests that exosomes containing miRNAs can be transferred from one cell to another. These results indicate that miRNA plays a regulatory role in paracrine signaling, and imply potential transfer of genetic information between cells [25]. Overall, miRNAs have been found in tissues, serum, plasma and other body fluids such as urine and saliva [25-27].

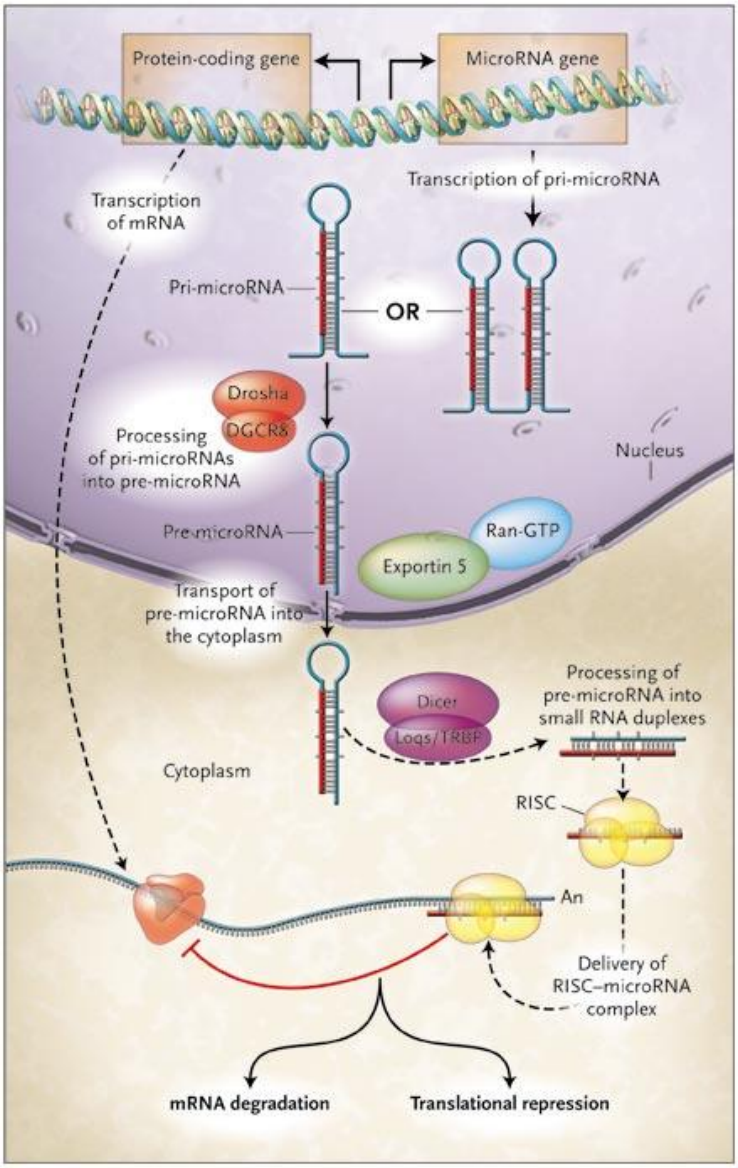


Fig.2 MicroRNA biogenesis. MiRNAs are processed by Drocha and Dicer with additional cofactors in the nucleus and cytoplasm, respectively, prior to being loaded into the RISC complex. The miRNA guides the RISC complex to target mRNA repressing protein synthesis either by repressing translation or mRNA degradation.

Reprinted from New Eng. J. Med., vol. 353, no. 17, pp. 1768-1771, Chen (2005), *MicroRNAs as Oncogenes and Tumor Suppressors,* with premission from New England Journal of Medicine.

1.1.3 MicroRNA clusters

Many miRNAs are encoded as polycistronic clusters [13]. Studies indicate that up to 50% of discovered miRNAs are encoded in this manner [11]. Functions of clustered miRNAs and their evolutionary origin remain unclear. However, there are indications that miRNA clusters are produced through tandem – and segmental gene duplication accumulating as a consequence of natural selection. These duplications have resulted in partially paralogous miRNA clusters located on different chromosomes [10].

MiRNA clusters are generally composed of two or more associated miRNAs encoded within a single RNA transcript [10, 28]. They frequently share sequence similarity and are co expressed with host genes [10, 11]. The members of a single miRNA cluster can be consistently expressed or differentially expressed. The mechanism underlying such variable expression is unknown.

MiRNA clusters frequently contain representatives from different miRNA families, meaning that miRNAs of a given cluster target multiple mRNAs. It has also been proposed that mRNA transcripts encoding co operating proteins, e.g., proteins involved in the same pathway, are typically targeted by miRNAs from the same polycistronic cluster [29]. Moreover, clustered miRNAs may regulate complex gene networks and signaling pathways, as more precise regulation is achieved by polycistronic clusters compared to individual miRNAs [10].

1.1.4 MicroRNAs in development

MiRNAs play an essential role in embryonic and tissue development [30]. Also, the expression of some mammalian miRNAs are tissue specific [31]. In ES (embryonic stem) cells, miRNAs have been implicated in sustaining cell identity. Later in development, miRNA are expressed in a dynamic fashion, often showing selective expression in differentiating cells [32-34].

The importance of miRNAs in development has been tested using Dicer knock-out mice. Dicer is essential for miRNA processing [35], and, therefore, a Dicer-deficient animal is unable to synthesis new miRNAs. Dicer deficient embryos die at an early embryonic stage and are depleted of pluripotent stem cells, supporting the crucial role for miRNA in embryogenesis and stem cell development [35, 36]. In all plant and animal species examined, defects in miRNA function have profound effects on development [37].

MiRNAs have been shown to regulate heart, brain, liver and muscle development, as well as organogenesis of skin and lung [36] [38] [31]. Specific roles of miRNAs have also been characterized in hematopoiesis [39].

1.1.5 MicroRNAs in disease

In view of the essential regulatory function of miRNAs [14, 40]; it is not surprising that miRNAs have been found to be involved in the pathogenesis of several diseases. For example, miRNAs are aberrantly expressed in cardiovascular disease, neurodegenerative disease (e.g. Alzheimer's disease), diabetes, kidney disease, and infectious disease [41-43]. Comparison of microRNA expression profiles in diseased and healthy tissue has shown the pathological state to be associated with altered expression of miRNAs [36].

MiRNAs have also been reported in several types of cancer [44]. Evidence indicates that miRNAs can function as tumour suppressor- or oncogenes, such miRNAs are called "oncomirs". If a target gene is an oncogene, loss of a corresponding miRNA, which function as a tumour suppressor, may cause synthesis of oncoprotein leading to tumour development [44]. On the other hand, amplification and overexpression of miRNAs functioning as oncogenes may cause translational inhibition of tumour suppressor mRNAs, also inducing tumour genesis. Many transcription factors regulate miRNA expression, and expression of some miRNAs are regulated by well-established tumour suppressor- or oncogenes, e.g. TP53, MYC and RAS [14, 16].

The abnormal expression of miRNA in different diseases may be caused by mutation in miRNA encoding genes or aberrant miRNA biogenesis. Therefore, miRNAs may serve as useful biomarkers [44]. Moreover, miRNAs could also serve as an alternative therapeutic target. A promising approach is to target disease-related miRNAs using anti-miRNA oligos (miRNA inhibitors) to knock-down overexpressed miRNAs. Another technique is systemic delivery of miRNA mimics for up-regulation of a selected miRNA [45]. New advances in delivery of miRNA inhibitors and mimics hold the promise of quickly translating our understanding of miRNA into useful therapy [44].

1.1.6 Techniques for studying microRNAs

MiRNAs can be studied using different approaches. An essential stage is the selection of which assay to use. Expression profiling is carried out using microarrays or deep sequencing [46, 47]. While microarrays have the ability to measure thousands of already sequenced genes, profiling of miRNAs by deep sequencing measures total abundance and allows for the discovery of new miRNAs [48]. Northern blotting, real-time PCR and *in situ* hybridization can be used to assay levels of individual miRNAs [46].

The list of known miRNAs is large and increasing. However, only a few regulatory targets are known. Prediction and validation of target mRNAs are necessary in understanding miRNA biology. Therefore, bioinformatic target prediction is often the first step towards defining function for a specific miRNA. Several computational methods and databases have been developed for this purpose (e.g. MiRanda, Target Scan and PicTar) [49]. Computational approaches are based mainly on miRNAs complementarity to their target mRNAs. Although target prediction criteria may differ, three principles are often involved: strong Watson-Crick base pairing of the "seed" sequence of the miRNA to a complementary site in the 3'UTR of the mRNA, conservation of the miRNA binding site, and the minimum free energy (MFE) of the miRNA-mRNA hetroduplex. Also, mRNA sequence features outside the target site can be significant, e.g. a good structural accessibility [49]. Based on theoretical considerations, a miRNA can have several hundred targets [50]. However, the resulting output of targets will depend on the algorithms used, and comparison of results from different algorithms is often useful.

Genes regulated by a miRNA may be identified using transcriptosome and proteome analysis. Verification of a miRNA target site can be performed by *in vitro* 3' UTR analysis. The functional relevance of a miRNA is best investigated by monitoring phenotypic changes in cultured cells or within an organism in response to changes in levels of a miRNA, so called reverse genetics [49]. Strategies for gain- and loss-of-function studies for specific miRNAs *in vivo* and *in vitro* have been developed. These studies include transfection experiments with miRNA mimics or anti-miRNAs, as well as transgenic animal models [45]. Thus, a combination of reverse genetics and computational prediction is useful when studying miRNA functions [49].

1.2 Why study microRNA expression during development of oral tissues?

To gain insight into the functional role of miRNAs during development it is necessary to accumulate information on their temporal and spatial expression. From studies of tissue- or cell-specific miRNA profiles, it may be possible to recognize miRNAs exhibiting prominent selective expression during proliferation, differentiation, maturation or apoptosis.

By studying this new layer of gene regulation in both *in vivo* and *in vitro* systems we can improve our understanding of the underlying molecular mechanisms of miRNAs. This knowledge can give an opportunity to further expand our understanding of molecular processes occurring both during normal and abnormal development.

The development of ectodermal organs such as scales, feathers, hairs, glands and teeth, is regulated by a complex dialog between and within epithelial and mesenchymal tissues [51]. At the start of this dissertational work, miRNAs had been shown to regulate skin morphogenesis [52], hair production [53], and mammary gland formation [54], but there were no published studies on miRNA expression in craniofacial organs such as teeth. MiRNA expression in oral tissues had not been address.

The development of oral tissues, e.g. teeth and salivary glands, involves several morphologically distinct stages, each controlled by genetic interactions involving growth factors, transcription factors, signal receptors and diffusible morphogens [51, 55]. Developing murine tooth and salivary gland, therefore, provide good models for studying miRNA expression during organogenesis. The major disadvantage of using the murine model is the small mass of collectable tissue and the consequently limited amounts of RNA which can be isolated.

The initial investigation of miRNA expression profiles required large scale microarray analysis. The first challenge was, therefore, to establish a protocol that could measure several hundred miRNAs using limited amounts RNA.

1.3 The miR-17-92 cluster

The results of our first study directed our attention to the members of the miR-17-92 cluster (Paper I). The miR-17-92 cluster is a polycistronic miRNA cluster located on chromosome 13 open reading frame 25 (C13orf25) in the human genome and on chromosome 14 in the mouse genome [56-58]. The primary transcript includes six tandem stem-loop hairpin structures that give rise to six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1 (Fig.3). These are encoded within an 800 base-pair region in the human genome [59]. The six miRNAs can be grouped into four miRNA families based on their seed sequence: the miR-17 family (miR-17 and miR-20a), the miR-18 family (miR-18a), the miR-19 family (miR-19a and miR-19b-1) and miR-92 family (miR-92a-1) [13, 57]. The oncogenic potential of miR-17-92 cluster was first described by He et al. The miR-17-92 cluster was the first oncogenic miRNA cluster identified and was named oncomir-1 [58].

The promoter region of miRNA encoding genes is highly analogous to those of protein-coding genes [9, 14]. The primary miR-17-92 transcript initiates from a consensus initiator sequence downstream of a nonconsensus TATA box [60]. In vertebrate genomes, a sequence upstream of the assumed transcriptional start site is highly conserved and contains an extensive CpG island, indicative of a core promoter region. Several transcription factors have been identified as activators of the miR-17-92 cluster; STAT3, c-Myc, n-Myc and E2F1 and E2F3 [60, 61]. Studies have also revealed that transcription of the cluster is repressed by p53 [57, 61].

The oncogenic potential of the miR-17-92 cluster has been observed both in human tumours and in animal models. The human genomic locus encoding these miRNAs, *13q31.3*, undergoes amplification in lymphomas and solid tumours [57]. The murine *miR-17-92* cluster is also a common insertion site in multiple types of retrovirally induced leukemia's [57]. However, in ovarian-, breast and skin cancer the cluster is deleted [62].

A complex interacting network comprising of Myc, E2F and the miR-17-92 cluster has been unraveled. The oncogenic function of the miR-17-92 cluster enables DNA-damaged cells to escape apoptosis [63]. The proliferative effect of the cluster can be partly due to miR-17 and miR-20 targeting the cyclin-dependent kinase inhibitor CDKN1A (p21) [61, 63].

Until recently it was unknown whether the oncogenic potential of the miR-17-92 cluster was caused by all components of the cluster or by a single member. However, recent reports have demonstrated that miR-19 is the major oncogenic constituent of the cluster *in vivo* and that the tumor suppressor PTEN is the major target of miR-19 [57, 61].

Members of the miR-17-92 cluster belong to different miRNA families and it is likely that they target unrelated mRNAs [59]. Therefore, a wide range of miRNA-mRNA interactions is possible, and consequently these interactions may result in substantial regulatory complexity. Mouse knock-out studies have shown members of the miR-17-92 cluster to possess specific, yet overlapping, functions [57]. So far, around 30 mRNAs targeted by members of the miR-17-92 cluster have been experimentally verified, including E2F1, Bim, HIF-1α and TNF-α. These genes encode proteins which are involved in regulation of cell cycle or apoptosis [61, 64]. However, the oncogenic effects of the members of miR-17-92 are probably mediated by regulation of multiple mRNA targets, many of which remains to be discovered.

Even though the miR-17-92 cluster has a well-known role in carcinogenesis, its physiological function remains uncertain. In 2007, Lu et al. demonstrated that the members of the miR-17-92 cluster are highly expressed in mouse embryonic lung and that the level of expression decreases as mice approaches maturity. The results also suggested that miR-17-92 promotes proliferation and inhibit differentiation of lung epithelial progenitor cells [38]. In a study by Ventura et al. loss-of –function of the miR-17-92 cluster caused postnatal death. The study also showed that the miR-17-92 cluster has a critical role during development of lung and heart [65].

Because of the documented role for the miR-17-29 cluster in development of lymphoma, lymphocyte development was studied in mice either lacking or overexpressing the miR-17-92 cluster. Several studies have suggested the cluster to be involved in development of B cells, T cells and monocytes. It has been suggested that the miR-17-92 cluster is essential for the survival signaling regulating progression from the pro-B cell to the pre-B cell stage. However, other hematopoietic cells, e.g., erythrocytes, monocytes, and T-cells are not affected by loss of these miRNAs. On the other hand, overexpression of the miR-17-92 cluster in lymphocytes *in vivo* leads to B-cell and T-cell proliferation and survival. The two tumor suppressors, Bim and PTEN, were identified as targets. The miR-17-92 cluster also controls monocyte development by inhibiting monocyte differentiation and maturation (reviewed in [62]).

In conclusion, the miR-17-92 cluster is commonly expressed in a variety of cell types and tissues, and effects of the miRNAs encoded in the miR-17-92 cluster will likely depend on the cellular context. However, pleiotropic effects of the miR-17-92 cluster remains unclear.

1.4 The miR-106a-363 and miR-106b-25 clusters

Ancient gene duplications have given rise to two miR-17-92 paralogs in mammals; the polycistronic miR-106a-363 cluster located on the X chromosome and the polycistronic miR-106b-25 cluster located on chromosome 5 in mice and chromosome 7 in humans [13, 59]. Each of these clusters contains miRNAs which are homologues to a subset of those of the miR-17-92 cluster [65]. The organization of miRNAs within these paralogs is conserved, and the sequence of each constituent miRNA is highly conserved across species [66]. Together, these three clusters contain 15 miRNA stem-loops, giving rise to 13 distinct mature miRNAs which belong to four miRNA families: the miR-106 family (miR-106a, miR-106b, miR-93, miR-17, miR-20a and miR-20b), the miR-18 family (miR-18a and mir-18b), the miR-19 family (miR-19a, miR-19b-1 and miR-19b-2) and the miR-92 family (miR-92a-1, miR-92a-2, miR-25 and miR-363) (Fig. 3) [59, 65].

The *miR-106-363* cluster lies directly at the 3'-end of the non-coding sequense *Kis2* in mouse [56]. The cluster encodes six miRNAs: miR-106a, miR-18b, miR-19b-2, miR-20b, miR-92a-2 and miR-363 (Fig.3) [65]. The *miR-106b-25* cluster is located in the intron 13 of the host gene *MCM7* in both humans and mouse, and are composed of three miRNAs: miR-106b, miR-93 and miR-25 [59, 66] (Fig.3). MCM7 is essential for the initiation of DNA replication in eukaryotes. MCM7 is transcriptionally regulated by E2F1 and MYC [63].

The miR-17-92 and miR-106b-25 clusters are abundantly expressed in many cell- and tissue types, while expression of the miR-106a-363 cluster is undetectable in most normal tissue examined [59, 66]. However, all three clusters are considered oncogenic and have been found to be up-regulated in various types of cancers [56, 66]. MCM7 and the miR-106b-25 cluster have been shown to co operate in tumour genesis, suggesting a functional interaction between an intronic miRNA and its host protein [66]. Thus, a single gene locus can have two independent and co operating oncogenic elements.

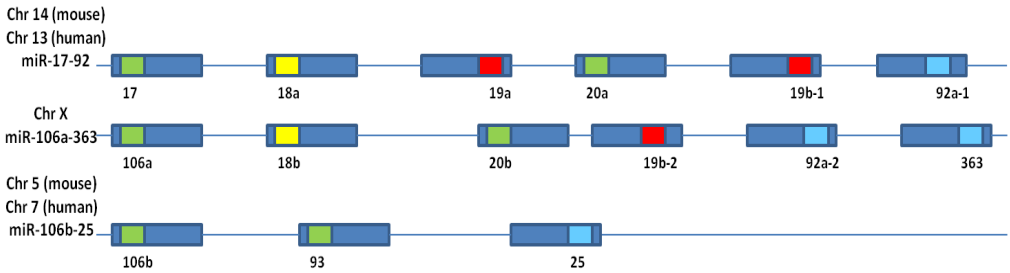
Given their sequence similarity, the three clusters are predicted to have highly overlapping targets. The miR-17-92 and miR-106b-25 clusters seem to be involved in analogous cellular functions such as the control of the cell cycle and TGFβ signaling [66]. E2F1 regulates the expression of both the miR-106b-25 and miR-17-92 clusters [60, 63, 67]. On the other hand, members of the miR-106b-25 and miR-17-92 clusters regulate E2F1 directly through a highly conserved binding site in its 3'-UTR [60]. In this manner a negative feedback loop between miR-106b-25/miR-17-92 and E2F1 is created (Fig.5) [63]. Also, miR-17, miR-20a, miR-106b and miR-93 repress p21 expression, whereas miR-92 and miR-25 inhibit BIM expression,

suggesting that members of the miR-106b-25 and miR-17-92 clusters inactivate the TGFβ pathway in a co-operative manner [57, 66].

Ventura et al. showed that levels of expression of the miR-17-92 and miR-106b-25 clusters are similar in mouse tissues [65]. However, only the miR-17-92 cluster is required for normal development, while the miR-106b-25 cluster appears to be dispensable. One possible explanation for this observation could be the difference between the two clusters; the miR-17-92 cluster encodes three miRNAs, miR-18a, miR-19a and miR-19b, which are not encoded in the miR-106b-25 cluster. Interestingly, a double knockout of both the miR-106b-25 and miR-17-92 clusters resulted in a more severely lethal phenotype, suggesting an additive effect of the miR-106b-25 cluster [59].

Li et al. showed that inhibition of expression of the miR-106 family contributes to typical features of senescent cells, e.g. increased resistance to apoptosis and activation of p21 [68]. While increased expression of miR-106a enhanced cellular proliferation, inhibition of miR-106a expression had no significant effects. The authors suggested that mechanism compensating for the loss of miR-106a could be present, e.g. a mechanism involving other members of the miR-106 family. So far, several targets for the miR-106 family have been experimentally verified, e.g. E2F1, Rbl2, BIM, p21, Mapk14 and Stat3 [61].

While several studies have focused on the expression and function of the miR-17-92 and miR-106b-25 clusters, the expression and function of the miR-106a-363 cluster remains unclear.



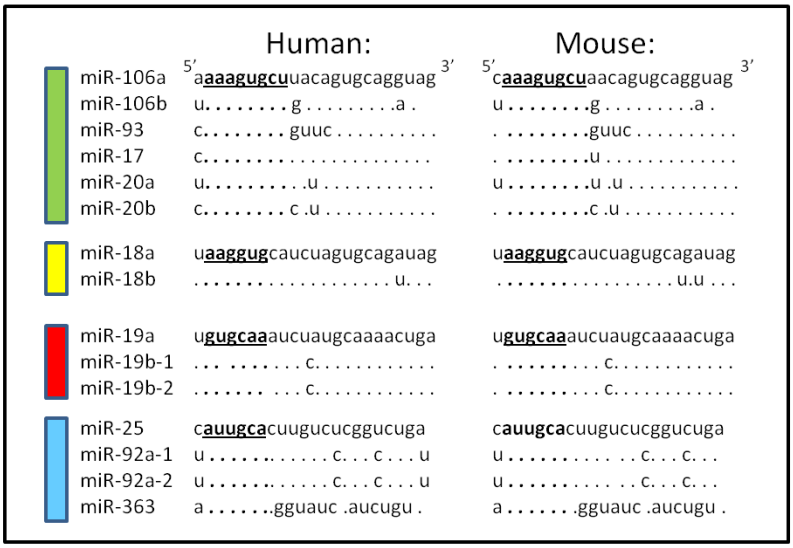


Fig.3. The genomic organization and primary transcripts of the human and mouse miR-17-92, miR-106a-363, and miR-106b-25 clusters. Based on their seed sequences the miRNAs of these clusters can be grouped into four families: the miR-106 family (miR-17, miR-20a/b, miR-106a/b, and miR-93) (blue); the miR-18 family (miR-18a/b) (yellow); the miR-19 family (miR-19a/b-1/2) (red); and the miR-92 family (miR-25, miR-92a-1/2, and miR-363) (green).

2 Study aims

The major aim of this thesis was to study miRNA expression during tissue development in mice. Such studies should reveal differences in expression profiles, perhaps providing a guide to their function.

The specific aims were:

1) Establish the miRNA expression profiles for two oral tissues; the murine molar tooth germ and the murine submandibular salivary gland during development (Paper I).

2) Investigate the levels of expression of the primary transcript and all mature members of the miR-17-92 cluster in a range of murine embryonic/young adult tissues, and cultured human oral keratinocytes, and cultured human squamous carcinoma cells (Paper II).

3) Investigate the level of expression of the primary transcripts and all matured members of the miR-17-92, miR-106a-363 and miR-106b-25 clusters following transfection of miR-363* mimic in cultured human squamous carcinoma cells (Paper III).

3 Summary of results

3.1 MicroRNA expression in developing murine tooth, salivary gland, and liver (Paper I)

A total of 112 different miRNAs were detected in developing murine tooth, salivary gland and liver. The expression profiles of 76 miRNAs detected in tooth germ at E15.5, P0 and P5 were established, together with the expression profile for the 88 miRNA found in submandibular salivary gland (SMG) at E15.5, P0, P5 and P25.

To validate the results, liver was used as a control tissue. This screening led to the identification of 82 miRNA expressed in liver, 48 of which were expressed at both time points investigated (E15.5 and P25). Several of these miRNAs had earlier been identified in mouse liver [69]. Also, 15 miRNAs appeared tissue-specific.

Among the 112 miRNA identified, 52 miRNAs were detected in all tissues examined. Among these, five of the members of the miR-17-92 cluster were identified (miR-17, miR-18a, miR-19b, miR-20a and miR-92a). The result revealed these miRNAs to be highly expressed in embryonic tissues and that their expression decreased during development.

3.2 Expression of the members of the miR-17-92 cluster in developing murine tissues (Paper II)

Microarray results obtained for miR-17, miR-18a, miR-19b, miR-20a and miR-92a were verified using real-time PCR. The data were further extended with additional tissues (lung, kidney, heart, brain (cerebral hemisphere), skin and small intestine) to investigate whether the pattern of expression of the members of the miR-17-92 is tissue specific or universal. The real-time PCR results showed that all members of the miR-17-92 cluster exhibited decreased expression at a postnatal stage (P25) compared to embryonic stages (E13.5 and E15.5).

To further extend mapping of expression of members of the miR-17-92 cluster, liver, lung and SMG were selected for additional analysis. The miR-17-92 cluster already had an established role in lung development [38], while the role of the cluster in liver and SMG was unexplored. The expression of all members of the miR-17-92 cluster was, therefore, measured across a selected range of developmental time-points in the three tissues (E11.5, E13.5, E15.5, E17.5, P0, P5 and P25).

In liver, the expression of all members of the cluster decreased with increasing time of development. Also, in the SMG reduced expression with progressive development is a common trait for all members of the miR-17-92 cluster. However, the pattern of expression observed in lung was somewhat different from that found in liver and SMG. In lung, the lower level of expression for miR-19b, miR-20a and miR-92a are found at E11.5, P0 and P25. The higher level of expression was found at E13.5 and E15.5. Interestingly, miR-17 show a higher and more stable level of expression in embryonic lung compared to the other two tissues.

Finally, the levels of expression of all members of the cluster were monitored in mouse embryos at E8.5 and E11.5. The results suggested that the level of expression of five (miR-17, miR-18a, miR-19a, miR-19b and miR-20a) of the six miRNAs was increased at E11.5 compared to E8.5, while that of miR-92a was significantly decreased.

To investigate whether the miR-17-92 cluster exhibited similar patterns of expression in embryonic tissues and in carcinoma cells we monitored the level of expression of all members of the cluster at selected passages of cultured, human squamous carcinoma cells (E10). In cultured carcinoma cells no decrease in expression was observed between P6, P15 and P25. Rather, there was a significant increase in levels of expression.

Further, to illustrate a potential association between proliferation and expression of miRNA encoded in the miR-17-92 cluster, we also measured expression of all members of the cluster in cultured, primary oral keratinocytes at selected passages. In two out of three cultures examined a consistent decrease in levels of expression of all members of the miR-17-92 cluster was observed at the higher cell passage number. Overall, levels of expression of individual miRNAs in cultured keratinocytes were more like those found in intact tissue.

3.3 Expression of the miR-17-92 primary transcript in developing murine tissues (Paper II)

The miR-17-92 cluster is a poly-cistronic transcript containing six miRNAs that are coordinately expressed, although the levels of resulting mature miRNAs differ. To investigate whether the level of expression of the primary miRNA transcript reflects that of the resulting mature miRNAs we measured the level of expression of the primary transcript, pri-miR-17-92, in mouse liver, lung and SMG, and in cultured human primary oral keratinocytes and cultured human oral squamous carcinoma cells at various time-points/cell passages.

To this end both pri-miR-17 (5'-flank of the gene) and pri-miR-92a-1 (3'-flank of the gene) were measured using real-time PCR. Mir-17, miR-18a, and miR-19a are located towards the 5'-flank of the gene, while miR-19b, miR-20a, and miR-92a are located towards the 3'-flank of the gene. The level of the primary transcript decreased during development in all tissues examined, and no significant differences between the 5'- and 3'-end were observed. In general, the level of expression of the primary transcript correlates with the expression of the matured miRNAs. The same correlation was observed with cultured human primary oral keratinocytes.

In cultured human squamous carcinoma cells (E10) no significant increase between P6, P15 and P25 were observed. The level of expression of the primary transcript and those of resulting corresponding miRNA do not, therefore, correlate in carcinoma cells.

3.4 Regulation of expression of microRNAs encoded by paralogous polycistronic clusters (Paper III)

The three paralogous clusters miR-17-92, miR-106a-363 and miR-106b-25 collectively give rise to 15 mature miRNAs (miR-106a, miR-106b, miR-17, miR-18a, mir-18b, miR-19a, miR-19b-1, miR-19b-2, miR-20a, miR-20b, miR-25, miR-92a-1, miR-92a-2, miR-93 and miR-363). All of these 15 miRNAs have an accompanying star stand species considered as a passenger strand. However, star strand miRNAs have lately been shown to be of regulatory relevance [70]. When screening cultured human oral keratinocytes, high levels of expression of the star strand species of miR-363, miR-363*, were observed. On the other hand, miR-363* was barely detectable in cultured human oral squamous carcinoma cells (E10). To investigate the function of miR-363* we, therefore, transfected E10 cells with miR-363*

mimic or scrambled control. The results showed decreased density of E10 cells and formation of cell colonies.

Microarrays were used to monitor the miRNA expression in E10 cells transfected with miR-363* -, miR-19a -, miR-20b mimic or scrambled control. In cells transfected with miR-363* mimic 27 miRNAs exhibited significantly lower level of expression. Only one miRNA molecule displayed significant increase: miR-485_5p. With miR-19a - or miR-20b mimic the majority of miRNAs affected showed increased level of expression.

Both the microarray and the real-time PCR results show that the level of expression of all members of the miR-17-92 cluster was decreased in cells transfected with miR-363* mimic compared to scrambled control. Also, all members of the miR-106b-25 cluster exhibited decreased levels of expression in the cells transfected with the miR-363* mimic compared to scrambled control. However, miR-106a was the only detectable member of the mir-106a-363 cluster in E10 cells. The level of expression of miR-106a was significantly decreased in cells transfected with miR-363*-mimic. In cells transfected with miR-19a, miR-20b and miR-92a mimics, effects on levels of expression of the different mature members varied depending on the mimic used.

Real-time PCR was used to measure levels of expression of the miR-17-92, miR-106a-363 and miR-106b-25 primary transcripts following transfection of miR-363* -, miR-19a -, miR-20b -, or miR-92a mimic, or scrambled control. Assays selective for pri-miR-17, pri-miR-106a or pri-miR-106b (5'-flank of the gene), and pri-miR-92a-1, pri-miR-92a-2 or pri-miR-25 (3'-flank of the gene) were used.

The level of expression of the miR-106b-25 cluster was 5-6-fold higher than that of the miR-17-92 cluster. For both clusters the assays based on the 3- end constantly showed much higher levels of expression compared to the 5'-end. The miR-106a-363 cluster was not detectable. In cells transfected with miR-363* mimic the level of expression of the miR-17-92 and the miR-106b-25 primary transcripts were significantly decreased. In cells transfected with miR-19a -, miR-20b -, or miR-92a mimic the level of expression of the miR-106b-25 primary transcript was increased. The level of expression of the miR-17-92 primary transcript was increased in cells transfected with miR-19a mimic, while no significant change in expression were observed in cells transfected with miR-20b - or miR-92a mimic.

4 General discussion

4.1 Methodological consideration

It is technically challenging to perform high specificity expression profiling of miRNAs due to their short length (~ 20 nt), heterogeneous GC content, lack of common sequence features (i.e., poly-A-tail), and the fact that the target sequence is present both in the pri-miRNA, the pre-miRNA and the mature miRNA (Fig.2) [71]. Also, distinct miRNAs of the same family may differ by only a single nucleotide [72]. Below some of these aspects are briefly discussed.

4.1.1 Isolation of small non-coding RNAs

The quality of RNA is important in miRNA microarray- and real-time PCR experiments [73]. Small RNA makes up ~0.01 % of all RNAs [72]. Therefore, the direct use of total RNA for miRNA assays will limit sensitivity. At the project onset in 2006, there was poor availability of commercial miRNA isolation kits. We were, therefore, confronted with the problem of isolating RNA enriched with respect to small RNAs. We, consequently, had to combine different extraction methods.

Column-based isolation kits provide binding of small RNA molecules (<200 nt) to a silica-based filter in presence of ethanol, e.g., the miRNeasy Mini Kit (Qiagen). However, to achieve optimal results with the miRNeasy Mini Kit, miRNA precursors should also be removed. Appreciable enrichment can be accomplished using the FlashPAGE Fractionator (Applied Biosystems), a device for rapid column gel electrophoresis, which efficiently facilitates isolation of small nucleic acids shorter than 40 nt. Unfortunately, this method is both relatively costly and time-consuming. On the other hand, in 2006 it did provide an efficient alternative to even more time-consuming polyacrylamide gel electrophoresis (PAGE) [72].

Lately, alternative column-based methods for isolation of miRNA enriched fractions, e.g., the mirPremier microRNA Isolation Kit (Sigma), have emerged. Unlike the miRNeasy Mini Kit, the mirPremier microRNA Isolation Kit yields fractions containing high-purity small RNA, essentially without contaminating large RNAs.

The protocol selected for isolation of RNA for microarray analysis must be chosen with the labeling procedure in mind [72]. Indirect labeling procedures usually involve the use of

enzymes and are, therefore, sensitive to polluting organic molecules. As a consequence phenol-based extractions should not be used.

4.1.2 MicroRNA microarrays

MicroRNA microarray technology is, as mRNA microarrays, based on nucleic acid hybridization between target molecules and their analogous complementary DNA oligonucleotide probes [47]. The short length of miRNA makes specific hybridization more difficult compared to mRNA. The probes are chosen, not because they possess ideal hybridization sequence, but because they are the only available sequences [74]. As individual miRNAs have a heterogeneous GC content, the predicted melting temperature (Tm) may span a relatively large range. Hence, it is not possible to select optimal probe sequences with common melting temperatures or guanine-cytosine content, and this fact can result in non-optimal hybridization specificity [74]. Therefore, the hybridization temperature is selected to achieve a compromise between specificity and sensitivity [46, 47, 72]. MiRNA arrays will, consequently, most likely not discriminate between members of miRNA families since their sequences are highly homologues [21]. This problem has been addressed using, e.g., control spots (negative or positive), spike-in controls or internal controls [72]. Alternatively, LNA (locked nuclear acids) probes, which provide higher annealing affinities, are used [47]. Further, replicate spotting of the same probe on an array can increase the precision of the measurements, and diminish problems caused by random signal noise.

Microarray assays are based on measurements of fluorescence signals with both a lower and an upper detection level [74]. A sample of RNA likely contains a wide range of miRNAs concentrations. MiRNA at a low concentration may be found below the threshold level of detection, while miRNAs present at very high concentrations may cause saturated hybridization signals. It is, therefore, not surprising that different platforms yield somewhat different results [74]. It is, consequently, particularly important to establish a lower threshold of detection prior to statistical analysis.

To achieve reliable results, microarray data must be background subtracted and normalized before being analyzed for genes that are differentially expressed [72]. Normalization is usually performed to remove dye labeling bias and differences in hybridization and scanning [72]. However, microarrays should in principle not be used for a quantitative measurement, but as a tool to map miRNA expression in two different samples.

When establishing a new protocol, a well known sample/tissue can be used to verify the method. In our first study we used liver as control tissue. Liver tissue had already been screened for miRNAs, several of which had also been verified by an alternative assay [69]. In general, microarray data should always be validated by an alternative assay [72]. In our case real-time PCR was used.

In our first study the oligonucleotide microarray printed at the Microarray Core Facility, The Radium Hospital, Oslo, Norway using the *mir*Vana miRNAProbe Set v.1.3 (Ambion Inc., TX, USA) was utilized together with the NCode labeling Kit (Invitrogen, CA, USA). These microarrays represented a comprehensive selection of human, mouse, and rat miRNAs from Sanger miRBase (272 miRNA probes). These were made of amine-modified DNA oligonucleotides 42-46 nt long. The probes contained an 18–24 nt sequence that target specific known human, mouse, or rat miRNAs, plus spacer and attachment sequences for coupling to the surface of the microarray slide. The disadvantage with these arrays is that orthologous sequences may be detected. The *mir*Vana miRNA Probe Set also included probes targeting an exclusive set of, at the time newly identified, human miRNAs, called Ambi-miRs. However, signals from these probes were not included in our analysis as we were studying murine tissues. All sequences were spotted in triplicates in each array. To validate specificity of the hybridization conditions, spike-in controls were used.

The microarrays from the Microarray Core Facility, The Radium Hospital, Oslo, Norway, later became unavailable. We, therefore, switched to Human miRNA OneArray™ Microarray v2 printed produced by Phalanx, Palo Alto, CAL., USA, in our third paper. These arrays contained 100% of Sanger miRBase v15 miRNA content, e.g. 1087 unique miRNA polynucleotide probes. All sequences were printed in triplicates. Probes on these arrays contained miRNA-specific sequences and a proprietary spacer designed to enhance the hybridization sensitivity to miRNAs. The NCode labeling kit (Invitrogen, CA, USA) could not be used in combination with these arrays due to high background labeling. Therefore, the ULS (universal linkage system) microRNA labeling kit (Kreatech Amsterdam, Netherlands) was utilized. According to the Phalanx hybridization protocol, USL labeled miRNA hybridization should be carried out at 37 °C. In our case, however, hybridization at 37 °C gave poor hybridization specificity. Therefore, hybridization was carried out at 41 °C. This temperature provided high specificity as well as high sensitivity. To validate our microarray results we used real-time PCR.

The great advantage of microarrays is the ability to measure expression of hundreds of genes in one single experiment. However, in the future microarray technology will face competition from the latest generation of very high throughput sequencing devices, which are predicted to make the microarray technology outdated. With these instruments sequencing of the complete miRNA transcriptome in various organisms is possible [48]. This method is not hampered by variability in melting temperatures, co expression of nearly identical miRNA family members or post-transcriptional modification.

4.1.3 Real-time PCR

Hybridization procedures may, as mentioned earlier, have limited specificity for mature miRNAs. Therefore, real-time PCR is the method of choice for validating microarray results. Expression profiling of miRNAs by real-time PCR have four main stages; cDNA synthesis, selection of primers (primer design), detection of amplified product and data normalization [71].

cDNA synthesis can be carried out by reverse transcription of miRNAs using stem-loop or linear miRNA specific primers, or by tailing RNAs [71]. The specificity and sensitivity of real-time PCR assays depend on primer design [46]. Stem-loop primers are designed to bind exclusively to the 3'-end of the miRNA. They include a double-stranded part, a loop, and short, single-stranded part contain the primer binding sequence [71]. There are several advantages to using stem-loop primers: 1) the annealing of a short RT primer to the 3'-portion of the miRNA provides improved specificity, 2) the double-stranded stem structure inhibits primer binding to pre-miRNAs, 3) the sequence of bases in the stem structure enhances the stability of the miRNA-DNA hetro-duplex leading to higher RT efficiency for short primers and 4) the unfolded stem-loop structure adds sequences downstream of the miRNA resulting in a longer RT-product [75]. We, therefore, utilized stem-loop primers in our studies (Applied Biosystems, CA, USA).

The principle of real-time PCR is based on detection of a fluorescent reporter molecule whose signal intensity is proportional to the amount of DNA present [71]. Two technologies exist for detection of microRNA using real-time PCR: SYBR Green I and TaqMan probes [71]. The TaqMan assay is more specific, as SYBR Green binds all double-stranded cDNA while the TaqMan probe binds specifically to the target cDNA sequence. Another disadvantage of SYBR Green-based assays is the production of false-positive signals from non-specific reaction products, and from primer-dimers or contaminating DNA [71].

The TaqMan probe hybridizes to an internal stretch of the amplicon and contains a fluorescent reporter and quencher. The close proximity of the fluorescence reporter to its quencher molecule prevents emission of the fluorescence. When the Taq polymerase reaches the 5'-end of the TaqMan probe, the exonuclease activity of the Taq polymerase hydrolyses the probe, and the quencher no longer remains close to the fluorescence reporter [75]. In consequence, a fluorescence signal proportional to the amount of PCR product is generated. The TaqMan microRNA assays (Applied Biosystems, CA, USA) combine use of stem-loop primers for cDNA synthesis and TaqMan PCR, providing specific and sensitive miRNA assays.

All real-time PCR data should be normalized when comparing results from different samples. The purpose of normalization is to remove as much technical variation as possible between samples [76]. To correct for sample-to-sample variation, e.g., amount of starting template and RNA quality, real-time PCR data is commonly normalized to an endogenous control (EC) gene. The EC should be stably expressed across all samples analyzed. Also, a normalizer molecule should have similar physical and chemical properties to that of miRNAs [76]. As the extraction efficiency of miRNA from samples is very different from longer RNAs, the normalizer molecule should be selected from a panel of small RNAs. However, an miRNA EC suitable for every tissue type and developmental stage has not yet been identified [77]. As far as we are aware of, there is no report on the validation of miRNA ECs for expression profiling of developing murine tissues. We evaluated several small nucleolar RNA genes. But, unfortunately, none of these were found to be consistently expressed (e.g. sno135, Applied Biosystems).

Precise experimental reason for selection of small nucleolar RNA genes as EC, e.g. RNU48 and U6, is lacking in a lot of publications. In fact, U6, together with S5, was shown to be the two least stable RNA species in a panel of 12 RNA targets evaluated across 13 normal human tissues [76]. Therefore, miRNA expression studies utilizing real-time PCR should begin with careful selection of appropriate ECs for normalization to ensure accurate quantitation. Ideally, a set of controls is chosen for the target cell, tissue, or treatment, as no single control can serve as a universal endogenous control for all experimental condition. However, the effort, cost, and sample requirements necessary for correct experimental selection of miRNA normalizers renders this impractical to achieve. In our mouse experiments, we utilized an in-house control to normalize between plates. The in-house control entailed the use of RNA from E10 cells (P) and the RNU48 assay.

Using the TaqMan method (Applied Biosystems, CA, USA) sample-to-sample variation is difficult to control as cDNA synthesis from miRNA and EC is carried out as separate reactions. Therefore, normalization against the EC does not directly correlate for the amount of starting template, RNA quality or the primer efficiency during cDNA synthesis. Nevertheless, we used RNU48 as EC in our cell culture experiments. Our data showed, however, that the same relative differences were detected with or without normalization against RNU48 suggesting sample to sample variation to be minor.

The rapid increase in number of miRNAs renders classical real-time PCR inefficient for expression profiling, and it is probably best used as a tool for validation.

The miR-17 assay

With cultured human squamous carcinoma cells, different levels of expression of miR-17 were reported in paper II and III (higher levels reported in paper III). To investigate possible reasons for the discrepancy, various batches of the miR-17 assay-kits (Applied Biosystems) were tested using tissues from different stages of development and two carcinoma cell lines (E10 and C12). The results indicated that these effects were due to variations between different batches of miR-17 assays kits, the more recent batches yielding the higher levels of expression.

4.1.4 Experimental design and statistical analysis

In all mouse experiments tissues/organs from at least three litters or three young adults were used. In cell culture studies at least three separate transfections were carried out. Further, all biological replicates were analyzed using three technical replicates. Studying expression trends a sample size of ≥ 3 was regarded as adequate. Because of the small samples size, and non-normal distribution, the non-parametic Mann-Whitney test was used for analysis of real-time PCR data. For microarray analysis ANOVA was used [74].

4.2 MicroRNA expression during development

Since their discovery in the early 1990s, miRNAs have been detected in an extensive range of tissues. Also, miRNAs are shown to be involved in almost every aspect of cellular activity [78, 79]. It is, therefore, not surprising that our results demonstrate that miRNAs are abundantly expressed during development of the murine first molar mandibular tooth germ, SMG and liver (Paper I). Numerous studies have demonstrated that miRNAs are essential for normal mammalian development [36, 79]. The essential role of miRNA during development is supported by the results observed with targeted inactivation of Dicer, which results in loss of processed miRNAs and accumulation of miRNA precursors [35]. However, these studies do not provide insights into the spatial and temporal expression, or biological functions of individual miRNAs. Mapping the expression of miRNAs in different tissues is important for understanding how they interact with target mRNAs to form the complex networks that regulate development.

While many miRNAs are ubiquitously expressed, some are expressed in a tissue-specific manner [31, 80]. Several studies have shown that miRNAs are necessary for proper skeletal and cardiac muscle development and function [31]. The muscle specific miR-1, miR-133 and miR-206 are perhaps the most studied and best-characterized miRNAs to date. Studies have revealed that these miRNAs are regulated by myogenic transcription factors. The miRNAs may function to "fine-tune" the output of the transcriptional networks, resulting in precise cellular responses to developmental needs. However, even though miR-1 and miR-133 are specifically expressed in both skeletal muscle and heart, their abundance varies during development (reviewed in [31]). Therefore, miRNA profiling of different tissues and organs during development, together with functional studies of individual miRNAs, can supply essential knowledge concerning their regulatory roles.

4.2.1 MicroRNA expression in the developing murine molar tooth and submandibular salivary gland

In the developing embryo, organs arise from their respective germ cells through reciprocal interactions between the epithelium and mesenchyme [81]. This also includes teeth and salivary glands [51, 55]. Organ development is strictly controlled by genetic programs which regulate morphogenesis, proliferation and cell differentiation [81]. Since miRNAs are thought

to regulate up to 50% of all protein-coding genes, they have the potential to assist in the countless regulatory mechanisms that occur during development [14].

The results from our first paper illustrate that a total of 112 miRNAs are expressed during development of murine tooth, SMG and liver. Out of these, 52 are expressed in all three tissues, indicating an extensive universal expression of miRNAs (Paper I). However, these tissues/organs are relatively heterogeneous, consisting of an extensive range of undifferentiated and differentiated cells. The miRNA profile will likely vary between different cell types, thus, the results represent the average levels of expression of miRNAs expressed in the various cell types in these tissues.

Our results also indicate that several of the miRNAs detected in tooth, SMG and liver are tissue specific (Paper I). However, later studies have revealed that some miRNAs considered tissue specific for tooth germ and SMG are also expressed in liver, e.g. miR-200b and miR-218 [82, 83]. Also, miR-429, considered tissue-specific in SMG, has later been detected in teeth [84]. These observations most likely reflect inadequate sensitivity in microarray assays and are, therefore, false-negative results. However, several of the "tissue-specific" miRNAs were detected at a very low level in their respective tissues, e.g. miR-133a, miR-206 and miR-195 in the tooth germ; miR-150, miR-28 and miR-341 in the SMG; and miR-381, miR-1 and miR-7 in the liver (Paper I). A relatively low hybridization temperature could have yielded high sensitivity but decreased specificity leading to false-positives. As miR-133a and miR-1 are described as a muscle-specific miRNA, the latter is more likely [31]. Thus, in conclusion, only a few miRNAs appears to truly be tissue-specific.

MiRNA expression during development is, however, precisely controlled in a time-dependent manner [80]. Our results show that a total of 31 miRNAs are expressed only during development (five of these are expressed only at the day of birth (P0)), whereas three miRNAs are expressed exclusively in mature liver. However, only seven of the 31 miRNA expressed during development are expressed in all tissues. This observation may indicate highly tissue-specific expression of miRNAs during development.

The function of microRNAs in murine oral tissues

The miR-200 family and miR-205

Epithelial mesenchymal transitions (EMT) describe the biological process that facilitates the conversion of epithelial cells into cells of a more mesenchymal phenotype, including the loss of cell-cell adhesion, loss of cell polarity, and the gaining of migratory and invasive properties [85]. EMT, therefore, plays a crucial role during development. Recent studies have shown that the members of the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c and miR-429) and miR-205 are essential regulators of differentiation and are expressed in epithelial cells [85, 86]. One study demonstrated that EMT is associated with down-regulation of miR-205, miR-141, miR-200, and miR-429, suggesting a negative regulatory role of these miRNAs on EMT. Induced expression of the miR-200 family in mesenchymal cells promoted mesenchymal epithelial transition (MET) [86]. The decreased expression of members of the miR-200 family, and miR-205, with EMT is reflected in lower levels of expression of these miRNAs in mesenchymal cells compared to epithelial cells [85]. In molar tooth germs miR-200b and miR-200c were detected and showed increased levels of expression from E15.5 to P0 (Paper I). Michon et al. detected the miR-200 family in incisor cervical loop epithelium at P5 [84]. In the SMG miR-200c were detected, and showed increased expression during development. However, members of the miR-200 family were not detected in liver (Paper I). MiR-205 was also detected in tooth germ and SMG, but not in liver. This miRNA exhibited increased expression in both tooth germ and SMG between E15.5 and P5. These levels of expression were verified using real-time PCR (Paper I). However, miR-205 was detected at a very low level in SMG at P25.

MiR-141 was identified in both tooth germ and SMG, showing increased level of expression between E15.5 and P0. In SMG levels of expression of miR-141 was decreased between P5 and P25 (Paper I). Because of cross-hybridization, it is not possible to discriminate between miR-200b and miR-200c. MiR-200c and miR-141 are transcribed from the same cluster [86], and showed similar patterns of expression in the respective tissues, although at different levels.

The let-7 family

Members of the let-7 family are highly conserved across species, suggesting that these miRNAs possess important regulatory functions. Among this family, let-7a has an identical sequence across species ranging from *C. elegans* to humans. Previously, studies have mapped the developmental expression patterns of let-7 in vertebrates, although a direct involvement of let-7 in development has not yet been demonstrated. Vertebrate let-7 family members are likely to have many roles, and it is technically demanding to knock out all members of the let-7 family in a single animal. However, an increase in let-7 expression at later developmental stages has been reported in many organisms. This increase is consistent with our findings. In the murine tooth germ and SMG an increased expression of several members of the let-7 family is found at later developmental stages (P0, P5 and P25) (Paper I). Let-7 is undetectable in human and mouse embryonic stem cells, but the level of let-7 increases in differentiated cells. Therefore, it has been proposed that a main role of let-7 is to promote differentiation. A relatively high expression of let-7 is sustained in various adult tissues. Conversely, decreased expression of let-7 levels has been found in many human cancers, reflective of the reversed embryogenesis occurring during tumour genesis (reviewed in [87]).

The function of microRNAs in tooth germ

In the murine tooth germ, the level of expression of miR-125b is gradually decreased during development (Paper I). In a study by Sonkoly et al., miR-125b was expressed in all organs studied, but the pattern of expression varied. However, the higher level of expression of miR-125b was detected in organs containing cells of ectodermal origin [88].

All members of the miR-199a/miR-214 cluster were detected in the murine tooth germ; i.e., miR-199a_3p, miR199a_5p and miR-214. Together with miR-125b, miR-214 and miR-199a_5p (miR-199a_AS) exhibited the higher levels of expression in tooth germ (Paper I). The expression of miR-214 was also verified using real-time PCR (Paper I).

In a study by Sehic et al. miR-214 were silenced *in vivo* in mouse tooth germ using anti-miR-214 [89]. The results illustrated that injection of anti-miR-214 altered the expression of seven other miRNAs including miR-199a_5p and miR-199a_3p. While the level of expression of miR-214 was significantly decreased, the level of expression of miR-199a_3p and miR-199a_5p were increased. The authors suggested that inactivation of miR-214 stimulated transcription of the miR-199a/miR-214 primary transcript. In anti-miR-214-treated molars the enamel showed evidence of hypomineralization, likely due to the decreased

expression of *Amelx* and *Enam*, encoding enamel matrix proteins [90]. In a study by Michon et al. conditional deletion of Dicer-1 in the epithelium resulted in a mildly abnormal tooth shape and enamel [84]. These results suggested that miRNA modulates tooth morphogenesis and ameloblast differentiation, possibly by fine-tuning signaling networks.

In another study by Sehic et al., the miRNA expression profile of the murine maxillary incisor tooth germ was screened at P0 and P10 using microarrays [91]. Comparing these results to our first paper shows that 16 of the miRNAs detected in maxillary incisor is also detected in first mandibular molar at P0 (Paper I). However, the majority of the miRNAs detected by Sehic et al. were not present on the miRVana v1.3 arrays (Ambion). On the other hand, while Sehic et al. utilized only the incisal part of the incisor, we used the whole first molar tooth germ. Therefore, it is impossible to perfectly compare the miRNA expression in these studies. On the other hand, the continuously growing incisor exhibits all stages of tooth formation along its inciso-apical axis. For instance, at P0, enamel matrix secretion has started in both the incisor and the first molar, indicating the same processes to be active in both tooth germs at this stage [92]. Our results were also confirmed by Cao et al., in that study miRNAs were shown to control tooth epithelial stem cell differentiation [93].

The function of microRNAs in the SMG

We are aware of only two published reports on miRNA expression in murine SMG. In addition to our study, a study by Hayashi et al. show that miR-21 are involved in branching morphogenesis of the murine SMG *in vitro* at E13.5 [94]. Our results show that miR-21 is also present in the SMG at E15.5, P0, P5 and P25 (Paper I). Moreover, Hayashi et al. detected 43 other miRNAs at E13.5, 26 of which were also detected in our study at one or more of the time-points investigated. Several of the miRNAs sequences detected by Hayashi et al. in the SMG were, however, not present on the miRVana v1.3 microarrays.

In general, our results indicate that miRNAs play important roles during murine SMG and tooth development. However, methods capable of unraveling events occurring in a single cell, or a small group of cells, will prove useful in future studies, e.g., *in situ* hybridization [95], or laze dissection studies.

4.2.2 Expression of the miR-17-92 cluster in developing murine tissues

To date, the miR-17-92 cluster is one of the best-characterized miRNA clusters [57]. Several members of the miR-17-92 cluster (miR-17, miR-18, miR-19a, miR-19b and miR-20) were identified in 2001 by Lagos-Quintana et al [96]. In 2002, Mourelatos reported that also miR-92a is encoded by the cluster [97].

In 2005, Hayashita et al. found the miR-17-92 cluster to be over-expressed in lung cancer [98]. Later, several reports have shown that miR-17-92 plays an important role during lung development [38, 99]. Members of the miR-17-92 cluster are among the more highly expressed miRNAs in mouse developing lung. Three members of the cluster: miR-17, miR-19b and miR-20a are present both in epithelium and mesenchyme of the embryonic lung [100]. The miR-17-92 cluster is also highly expressed in endothelial cells [101, 102], and contributes to development of heart and blood vessels [59, 62, 65]. We have shown that the miR-17-92 cluster is also abundantly expressed in the developing murine tooth, SMG and liver (Paper I).

In the developing lung, levels of expression of all members of the cluster have been found to gradually decline until they are barely detectable in the adult lung [38]. A similar trend was observed with developing murine tooth, SMG and liver (Paper I). In these tissues the members of the cluster are highly expressed at an embryonic stage, while their levels are diminished at postnatal- and young adult stages.

To provide an insight into the temporal expression of the members of the miR-17-92 cluster in different tissues, microarray data were validated and extended to additional developmental stages and tissues using real-time PCR (Paper II). The results show that also in murine skin, cerebral hemisphere, small intestine, kidney and heart, the members of the miR-17-92 cluster show higher level of expression at early embryonic stages compared to young adult stages. Therefore, the miR-17-92 cluster may be important for the development of phenotypically diverse tissues.

Functions of the miR-17-92 cluster

Results have suggested that miR-17-92 promotes proliferation of undifferentiated lung epithelial progenitor cells [38]. In the liver, all members of the miR-17-92 cluster were shown to have consistently higher level of expression at embryonic stages compared to postnatal stages (Paper I and II). The lower level of expression was found at P25. The expression of miR-122, a highly abundant, liver-specific miRNA, increases during development and the

higher level of expression of this miRNA is found in the fully developed liver [103]. These findings suggest that the expression of tissue-specific miRNAs increases as cells achieve their final identity. In parallel, expression of the members of the miR-17-92 cluster is decreased. These findings support the idea that members of the miR-17-92 cluster are highly expressed in proliferating, undifferentiated cells, where they may serve to maintain high rates of proliferation and likely suppress differentiation [59, 63].

To further investigate possible involvement of the miR-17-92 cluster in proliferation, we decided to measure the levels of expression of all members of the cluster at selected passages of cultured primary oral keratinocytes. A consistent decrease in levels of expression of all miRNAs was observed at the higher cell passage number for two out of three studied cultures, while the level of expression of miR-17, miR-19a, miR-19b, miR-20a and miR-92a were unchanged at the higher cell passage number in the third culture (Paper II). These results likely reflect biological variation as the keratinocyte cultures are derived from separate donors. However, the results indicate that the miR-17-92 cluster indeed promotes cell proliferation and show decreased expression in differentiated cells. Keratinocytes has been shown to differentiate in culture [104].

Carraro et al. have shown that miR-17, miR-20a and miR-106b (a member of the miR-106b-25 cluster) are involved in lung branching morphogenesis [99]. These miRNAs regulate translation of Mapk14 and Stat3. Over-expression of miR-17 was shown to cause increased cell proliferation. Cloonan et al. suggested that miR-17 has the ability to regulate cell cycle at the G1/S check point [105], and thereby, increasing rate of proliferation.

Interactions between the miR-17-92 cluster and transcriptionfactores MYC and E2F

MiRNA clusters produce multiple miRNAs from one single primary transcript and each of these may function independently. Therefore, cluster-derived miRNA may regulate numerous genes, making them important regulators of the complex molecular network controlling development [57]. The miR-17-92 encoding six miRNAs may, therefore, have an appreciable regulatory potential. So far, several targets of the miR-17-92 cluster have been verified [61].

MiRNAs encoded in the miR-17-92 cluster can be divided into four families (Fig.3). Interestingly, miRNAs belonging to the same family exhibit different levels of expression (Paper II). Therefore, members of the same miRNA family may have different functions, even though they have identical “seed” sequences and, therefore, should potentially have shared targets.

The functions of miRNAs are context-dependent, meaning that the function of a miRNA will depend on composition of the transcriptome, which will vary between different cell types [57, 106]. As a single miRNA can have several targets, its function will depend on the mRNA profile. The fact that the expression of miRNAs can be regulated via several other genes adds further to their regulatory complexity.

An interesting regulatory feedback loop exists between miR-17-92 and E2F (Fig. 5). Two members of the miR-17-92 cluster, miR-17 and miR-20, are known to repress the cell cycle regulator E2F1. On the other hand, E2F1 has been shown to activate transcription of the miR-17-92 cluster. E2F1 is one of the main drivers of the G1/S transition during the cell cycle. The miR-17-92 cluster is also transcriptionally activated by the proto-oncogene c-Myc. Since c-Myc also promotes E2F1 transcription it is likely that c-Myc fine-tune cell cycle progression via regulation of both miRNA and mRNA expression (reviewed in [63]). Control of the miR-17-92 cluster by E2F and c-Myc likely play an important role in cell proliferation during development.

4.2.3 Expression of the miR-106a-363 and miR-106b-25 clusters in developing murine tissues

The miRNA microarray results from our first paper may contain false positives due to a 10% mismatch. Therefore, considering the sequence similarities of the members of the miR-17-92, miR-106a-363 and miR-106b-25 clusters, there is a high probability that some of the mature paralogous miRNAs will bind to the same probe, e.g. miR-20a and miR-20b (Fig. 3). As a consequence, microarray results indicate that all members of the miR-17-92 and miR-106b-25 clusters are expressed at one or more of the developmental stages investigated (Paper I). Based on these assumptions, all members of the miR-106a-363 cluster, except for miR-363, are also expressed in the developing tissues (Paper I). The probe for miR-363 was not present on the miRVana arrays. However, several studies have shown that while the miR-17-92 and miR-106b-25 clusters are expressed in several different tissues, expression of the miR-106a-363 cluster have not been detected [59]. Therefore, detected members of the miR-106a-363 cluster may be due to cross-hybridization because of paralogous miRNA sequences in the miR-17-92 and miR-106b-25 clusters.

4.3 Expression of the miR-17-92, miR-106a-363 and miR-106b-25 clusters in cultured human squamous carcinoma cells

All members of the miR-17-92 cluster are expressed in cultured human squamous carcinoma cells (Paper II and III), although levels of expression of individual members vary. However, the results show that levels of all miRNAs encoded in the cluster increases with increasing number of cell passages (Paper II). These results contrast findings obtained with both developing murine tissues and cultured differentiating human oral keratinocytes (Paper I and II). However, in carcinoma cells, no increase in level of expression of the primary transcript was observed. Therefore, the elevated level of the mature miRNAs in later passages is likely caused by diminished degradation of the transcript.

Members of the miR-106b-25 cluster were also identified in cultured squamous carcinoma cells (Paper III). The level of expression of miRNAs encoded in this cluster also differed as we found that miR-93, miR-106b, and miR-25, were expressed at a low-, intermediate- and high level, respectively (Paper III). This result show that also in cultured squamous carcinoma cells the level of expression of mature miRNAs encoded in clusters is likely regulated post-transcriptionally.

In squamous carcinoma cells, four members of the miR-106a-363 cluster were detectable: miR-106a, miR-19b, miR-20b and miR-92a. However, miR-19b and miR-92a may originate from the miR-17-92 cluster. Therefore, miR-106a and miR-20b may be the only miRNAs derived from the miR-106a-363 cluster. The level of expression of miR-20b was, however, barely detectable. These results indicate that a polycistronic cluster, consisting of six potential miRNA, may give rise to a single functional, mature miRNA.

4.3.1 A possible role for miR-363*

Processing of the miRNA hairpin precursors (pre-miRNA) give rise to a double stranded small RNA duplex (~ 22 nt) (Fig. 2). Although both stands of the pre-miRNA duplex are produced in identical amounts, their accumulation is asymmetric (Fig. 5). Based on the thermodynamic stability of each end of this duplex, one of the stands is regarded as the biologically active miRNA. The other is considered as an inactive strand, called the miRNA* or passenger strand. Generally, the biological active strand of the duplex is selected for entry into the RISC complex [107], while the passenger strand is degraded.

However, recently, some miRNA* sequences were reported as mature functional miRNAs with abundant expression [70]. Also the miRNA/miRNA* ratio may vary dramatically between developmental stages [14, 107]. Interestingly, miRNA profiling of cultured human oral keratinocytes showed appreciable expression of miR-363*, a member of the miR-106a-363 cluster (and the star strand species of miR-363). However, in cultured human squamous cancinoma cells (E10) the expression of miR-363* was barely detectable. Considering this observation, and the fact that the miRNA* species is generally regarded as a passenger RNA [70], the function of which has not been examined in vertebrates, we decided to investigate the effects of miR-363* by transfection with miR-363* mimic in E10 cells.

Transfection with miR-363* mimic in E10 cells led to decreased cell proliferation and decreased level of expression of the miR-17-92 and miR-106b-25 clusters (Paper III). As miRNAs encoded in these clusters show high levels of expression in proliferating cells [63], the observed decline in level of expression of these miRNAs are consistent with the observed decrease in the number of cells. The decreased expression of miRNAs encoded in the miR-17-92 and miR-106b-25 clusters may cause diminished proliferation. However, as expression of the miR-17-92 and miR-106b-25 clusters are regulated by proteins regulating cell cycle, e.g. E2F and c-MYC, the decreased expression of these clusters, and the diminished cellular proliferation, may be caused by altered cellular level of such proteins.

Decreased cellular proliferation was also observed in cells transfected with miR-20b mimic (Paper III). The miRNA expression profiles were, however, markedly different. Therefore, miRNA-mediated mechanisms inhibiting cellular proliferation are likely also different.

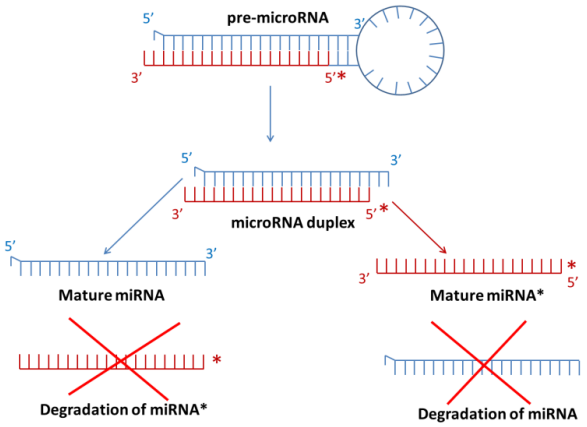


Fig. 4. Selection of the mature functional miRNA strand. The mature functional miRNA strand is generated from either the miR strand or miR* (star) strand. The passenger stand is degraded. Both the miR and miR* strand are potential mature miRNA sequences.

4.4 Regulation of transcription of genes encoding microRNA clusters

Nearly half of the loci for mammalian miRNAs are closely located, often in clusters, with a "master" promoter for the production of a single polycistronic transcription unit [9, 11]. Transcription of these miRNA clusters is probably strictly regulated.

Most pri-miRNAs are transcribed by RNA Poly II and contain the 7-methylguanylate cap at the 5'-end and a poly (A) tail at the 3'-end, a trait also characteristic of mRNAs [15]. Since most miRNA encoding genes are transcribed by RNAPII, they are believed to share the regulatory mechanisms of protein encoding genes. Promoters of miRNA encoding genes have regulatory sequences analogues to those of protein encoding genes, e.g., relative frequencies of CpG islands, TATA box, TFIIB recognition elements and initiator elements [9]. DNA-binding factors that regulate transcription of miRNA encoding genes are also similar to those controlling protein encoding genes. However, the transcriptional regulation of miRNA clusters is less readily studied because promoter regions of ncRNAs are often difficult to identify [9].

Since paralogous clusters are located on different chromosomes and produce separate transcripts, their transcription may be independently regulated. Consequently, functional differences between such clusters could be due to separate regulatory mechanisms. Therefore, paralogous miRNA clusters exhibiting non-synchronous expression may be subjected to independent regulation of transcription. However, some paralogous miRNA clusters on different chromosomes exhibit synchronous expression. These miRNA clusters may, therefore, have synchronous regulatory schemes and may consequently function in a cooperative manner (reviewed in [10]). In fact, Kim et al. reported that separate clusters can be co-expressed and have related functions [108].

Both the primary transcript and all mature members of the miR-17-92 cluster were detected in the developing murine tooth, SMG and liver (Paper II). Also, several members of the miR-106a-363 and miR-106b-25 clusters were detected in all tissues (Paper I). However, as mentioned earlier, the detection of members of the miR-106a-363 cluster may be due to cross-hybridization. In human squamous carcinoma cells the primary transcript and all mature members of the miR-17-92 and miR-106b-25 clusters were detected using real-time PCR, while miR-106a was the only detectable member of the miR-106a-363 cluster (Paper III). The miR-106-363 primary transcript was undetectable. Therefore, the miR-17-92 and the miR-

106b-25 clusters may by regulated by similar transcriptional elements, while the miR-106a-363 cluster is subject to a separate regulatory scheme. As previously discussed, transcription of the miR-17-92 is regulated by MYC and E2F. The miR-106b-25 cluster is also activated by E2F and MYC in parallel with its host gene, MCM7 [66, 109]. In turn, miR-17, miR-20a, miR-106b and miR-93 regulate expression of E2F, establishing a negative feedback loop (Fig. 5). However, expression of all three clusters are repressed by p53 [63].

Tanzer et al. described a duplication-degeneration-complementation model for the evolution of the miR-17-92 cluster which predicts that after gene duplication either one of the two paralogs is lost [13]. If both are retained, the two paralogs will evolve to perform complementary functions. The model further predicts that duplicate clusters containing redundant miRNAs should differ in their spatial and temporal pattern of expression.

We also observed a strong correlation between the level of expression of the primary transcript and the level of mature miRNAs. This relationship indicates that regulation of transcription is one mechanism regulating the level of the mature miRNA. Post-transcriptional regulation may serve to fine-tune the level of individual miRNAs.

Transfection with miR-363* mimic in cultured, human squamous carcinoma cells caused diminished expression of all members of the miR-17-92 and miR-106b-25 clusters. However, the level of expression of the respective primary transcripts was also decreased. These observations indicate that the miR-363*, a member of a paralogous cluster, can directly or indirectly regulate transcription of the miR-17-92 and miR-106b-25 clusters.

However, in cells transfected with miR-19a, miR-20b or miR-92a, correlation between the level of expression of the miR-17-92 and miR-106b-25 primary transcripts and the level of expression of their respective members were not found. While the levels of expression of the primary transcripts were increased, the levels of expression of some mature members were decreased. Consequently, our results show that cellular levels of miRNAs are regulated at two levels; transcriptionally and post-transcriptionally.

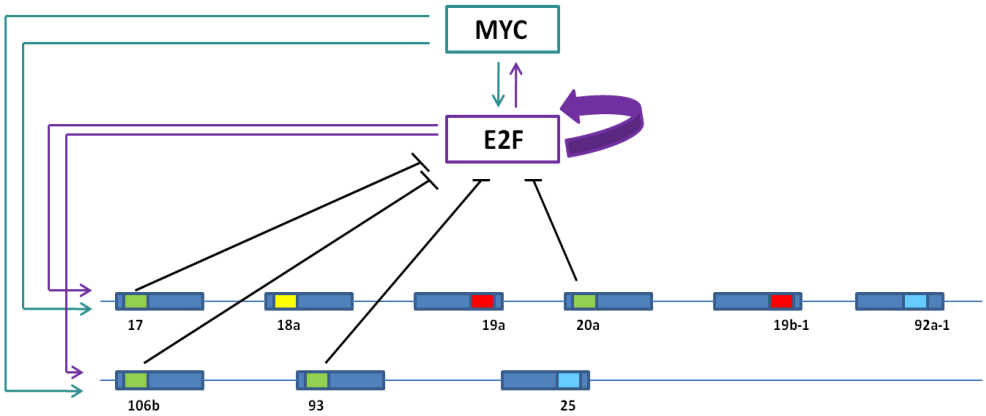


Fig.5 Summary of the interactions among the transcription factors Myc and E2F, and members of the miR-17-92 and miR-106b-25 clusters. All arrows indicate induction of gene expression. The hammerheads from miR-17, miR-20a, miR-106b and miR-93 to E2F indicate inhibition of translation or degradation of the mRNA.

4.5 Processing of polycistronic microRNAs into mature microRNAs

From the initial transcription of miRNA encoded genes in the nucleus to the synthesis of mature miRNA in the cytoplasm, their production may be subject to regulation at several levels. Both transcriptional and post-transcriptional mechanisms are likely involved [14].

Although miRNAs encoded in the miR-17-92 cluster are derived from a single polycistronic transcript, Lu et al. found that levels of expression of individual members of the miR-17-92 cluster vary in the developing lung [38]. In developing murine liver, SMG, tooth, skin, cerebral hemisphere, small intestine, kidney and heart, differences in levels of expression of members of the miR-17-92 cluster were also observed (Paper II). These findings indicate that levels of expression of the miRNAs encoded in the miR-17-92 cluster are regulated post-transcriptionally. Analogous results were found for miRNAs encoded in the miR-17-92 and miR-106b-25 clusters in cultured human squamous carcinoma cells (Paper II and III).

Our results show strong correlation between pattern of the expression of primary transcripts and the pattern of expression of the mature miRNAs (Paper II). Further, no difference in levels of expression between the 5'-end and the 3'-end of the transcript were found in the developing tissues, therefore, the proportional difference between the members of a miRNA cluster is most likely caused by post-transcriptional regulatory mechanisms. However, in human squamous carcinoma cells a significant difference between the 5'-end and the 3'-end of the transcript was found (Paper III). The assays based on the 3- end constantly showed much higher levels of expression. These findings suggest the 5'-end to be less stable than the 3'-end transcript.

The level of mature miRNAs encoded by the same cluster may result from differential processing in the nucleus by Drocha, selective transport into the cytoplasm by Expotin-5, or discriminating processing by Dicer in the cytoplasm (Fig. 3). Actually, RNA editing of several pri-miRNAs by ADAR (adenosine deaminase acting on RNA) can affect both Drocha and Dicer-mediated cleavage, leading to modification of sequence information causing altered miRNA processing efficiency [14].

Several reports have identified proteins that regulate processing either by interacting with Drocha or Dicer or by binding to the pri- or pre-miRNA [14]. As processing into mature miRNAs require binding of specific cofactors, the RNA sequences surrounding the miRNA,

e.g. stem-loop sequences, may be significant for processing of individual cluster-encoded miRNAs.

The activity of some of the regulatory cofactors is limited to specific miRNA families. For example, hnRNP A1, a multifunctional RNA-binding protein, binds specifically to the primary RNA sequence of pri-miR-18a before Drocha processing, indicating that cluster-encoded miRNAs undergo specific processing [110]. However, selection among family members may also take place as these are detected at different levels, e.g., miR-17 and miR-20a (Paper II and III).

Tertiary folding of the pri-miRNA transcripts may also regulate processing of clustered miRNAs. The consequence can be variable efficiency as regards processing of different regions of the primary transcript.

In conclusion, the processing of miRNAs derived from a single initial transcript is still poorly understood.

5 Conclusions

MicroRNA expression in developing murine tissues:

- MicroRNAs are abundantly expressed in murine first molar mandibular tooth germ and murine submandibular salivary gland, and their expression is highly dynamic; the miRNA profile changes extensively with time of development. Bioinformatics analysis suggests that miRNAs are "fine-tuning" the activity/quantity of mRNAs expressed during organogenesis.

Functional role of the miR-17-92 cluster:

- The levels of expression of microRNAs encoded in the *miR-17-92* cluster are consistently higher at early stages of development in phenotypically very different tissues.

- The level of expression of microRNAs encoded in the miR-17-92 cluster in cultured human oral kerationocytes at different passages may reflect ongoing keratinocyte differentiation.

Regulation and processing of microRNA clusters:

- Individual mature constituents of the miR-17-92 cluster exhibit different levels of expression in murine tissues, in cultured oral human keratinocytes and in cultured human squamous carcinoma cells, indicating post-transcriptional regulation of these miRNAs. Analogous results were found with members of the miR-106b-25 cluster in cultured human squamous carcinoma cells.

- In murine tissues, the expression pattern of the miR-17-92 primary transcript correlates with expression of the matured microRNAs encoded in the cluster. Post-transcriptional regulation may, therefore, be regarded as "fine-tuning" of levels of expression of individual miRNAs.

Functional role of miR-363*:

- Cultured human squamous carcinoma cells transfected with miR-363* mimic exhibited significantly decreased proliferation, and decreased expression of the miR-17-92 and miR-106b-25 primary transcripts and of all miRNAs encoded in these clusters. Therefore, levels of expression of polycistronically encoded miRNAs are influenced by the cellular level of a miRNA encoded by a paralogous gene. Moreover, miRNA*-species are likely functional and, thus, contribute to the miRNA regulatory network.

6 Future perspectives

Despite appreciable accumulation of knowledge concerning miRNAs during the last few years, information regarding function of miRNA during development is limited. Expression profiling has shown that these small RNAs are widely expressed in developing tissues/organs. However, most miRNA targets have not been experimentally verified. Therefore, improved experimental techniques enabling direct verification of targets are needed. Also, techniques suited to explore the impact of miRNAs on protein output is required, e.g. proteomics technology [111]. It is also important to study miRNA-mRNA interactions in a cellular context, as the relationship between miRNA and a target found in one type of cell may not be valid in another type of cell.

Our understanding of regulation of miRNA biogenesis is limited, especially biogenesis of clustered miRNAs. Therefore, studies focusing on regulation of expression of miRNA clusters and processing of their constituent miRNAs are important.

So far most work with miRNAs has focused on the role of individual miRNAs as regulators of translation. It is seems relevant also to study regulatory effects of combinations of miRNAs. MiRNAs encoded in a cluster are a likely candidate for such studies. Further, studies of concerted effects of miRNAs are essential if the therapeutic potential of miRNAs is to be exploited.

To further increase our understanding of miRNAs in developing murine tissues and the function of miRNAs encoded in the miR-17-92, miR-106a-363 and miR-106b-25 clusters, the following studies may be carried out:

- Elucidation of cellular locations of candidate miRNAs in developing murine molar tooth germ or submandibular salivary gland (*in situ* hybridization)
- Transfection or co-transfection of cells with miRNA mimics for remaining members of the miR-17-92, miR-106a-363 or miR-106b-25 clusters
- Transfection of cells with anti-miRNAs against miRNAs encoded in the miR-17-92, miR-106a-363 or miR-106b-25 clusters
- Screening the mRNA profile in cells transfected with miRNA mimics or anti-miRNAs to identify potential targets (microarrays). Such studies should be combined with appropriate methods, e.g., Western-blotting, to monitor changes in levels of proteins.

References

1. Mattick JS: **RNA regulation: a new genetics?** *Nature reviews Genetics* 2004, **5**(4):316-323.
2. Wright MW, Bruford EA: **Naming 'junk': human non-protein coding RNA (ncRNA) gene nomenclature**. *Hum Genomics* 2011, **5**(2):90-98.
3. Zhao S, Liu MF: **Mechanisms of microRNA-mediated gene regulation**. *Science in China Series C, Life sciences / Chinese Academy of Sciences* 2009, **52**(12):1111-1116.
4. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.
5. Moss EG, Lee RC, Ambros V: **The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA**. *Cell* 1997, **88**(5):637-646.
6. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: **The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans**. *Nature* 2000, **403**(6772):901-906.
7. Lee RC, Ambros V: **An extensive class of small RNAs in Caenorhabditis elegans**. *Science* 2001, **294**(5543):862-864.
8. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function**. *Cell* 2004, **116**(2):281-297.
9. Liang R, Bates DJ, Wang E: **Epigenetic Control of MicroRNA Expression and Aging**. *Curr Genomics* 2009, **10**(3):184-193.
10. Zhang Y, Zhang R, Su B: **Diversity and evolution of MicroRNA gene clusters**. *Sci China C Life Sci* 2009, **52**(3):261-266.
11. Olena AF, Patton JG: **Genomic organization of microRNAs**. *J Cell Physiol* 2010, **222**(3):540-545.
12. Miyoshi K, Miyoshi T, Siomi H: **Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production**. *Molecular genetics and genomics : MGG* 2010, **284**(2):95-103.
13. Tanzer A, Stadler PF: **Molecular evolution of a microRNA cluster**. *J Mol Biol* 2004, **339**(2):327-335.
14. Krol J, Loedige I, Filipowicz W: **The widespread regulation of microRNA biogenesis, function and decay**. *Nat Rev Genet* 2010, **11**(9):597-610.
15. Davis-Dusenbery BN, Hata A: **Mechanisms of control of microRNA biogenesis**. *J Biochem* 2010, **148**(4):381-392.
16. Zhang X, Zeng Y: **Regulation of mammalian microRNA expression**. *Journal of cardiovascular translational research* 2010, **3**(3):197-203.
17. Iorio MV, Piovan C, Croce CM: **Interplay between microRNAs and the epigenetic machinery: an intricate network**. *Biochim Biophys Acta* 2010, **1799**(10-12):694-701.
18. Fabian MR, Sonenberg N, Filipowicz W: **Regulation of mRNA translation and stability by microRNAs**. *Annu Rev Biochem* 2010, **79**:351-379.
19. Huntzinger E, Izaurralde E: **Gene silencing by microRNAs: contributions of translational repression and mRNA decay**. *Nature reviews Genetics* 2011, **12**(2):99-110.
20. Bartel DP: **MicroRNAs: target recognition and regulatory functions**. *Cell* 2009, **136**(2):215-233.

21. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP: **The microRNAs of Caenorhabditis elegans**. *Genes & development* 2003, **17**(8):991-1008.
22. Didiano D, Hobert O: **Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions**. *Nat Struct Mol Biol* 2006, **13**(9):849-851.
23. Breving K, Esquela-Kerscher A: **The complexities of microRNA regulation: mirandering around the rules**. *Int J Biochem Cell Biol* 2010, **42**(8):1316-1329.
24. Jeffries CD, Fried HM, Perkins DO: **Additional layers of gene regulatory complexity from recently discovered microRNA mechanisms**. *The international journal of biochemistry & cell biology* 2010, **42**(8):1236-1242.
25. Etheridge A, Lee I, Hood L, Galas D, Wang K: **Extracellular microRNA: A new source of biomarkers**. *Mutat Res* 2011.
26. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT: **Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection**. *Clin Cancer Res* 2009, **15**(17):5473-5477.
27. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K: **The microRNA spectrum in 12 body fluids**. *Clin Chem* 2010, **56**(11):1733-1741.
28. Xiang J, Wu J: **Feud or Friend? The Role of the miR-17-92 Cluster in Tumorigenesis**. *Curr Genomics* 2010, **11**(2):129-135.
29. Xu J, Wong C: **A computational screen for mouse signaling pathways targeted by microRNA clusters**. *RNA* 2008, **14**(7):1276-1283.
30. Stefani G, Slack FJ: **Small non-coding RNAs in animal development**. *Nature reviews Molecular cell biology* 2008, **9**(3):219-230.
31. Williams AH, Liu N, van Rooij E, Olson EN: **MicroRNA control of muscle development and disease**. *Curr Opin Cell Biol* 2009, **21**(3):461-469.
32. Pauli A, Rinn JL, Schier AF: **Non-coding RNAs as regulators of embryogenesis**. *Nature reviews Genetics* 2011, **12**(2):136-149.
33. Kiefer JC: **microRNAs under the microscope**. *Developmental dynamics : an official publication of the American Association of Anatomists* 2006, **235**(3):846-853.
34. Foshay KM, Gallicano GI: **miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation**. *Developmental biology* 2009, **326**(2):431-443.
35. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ: **Dicer is essential for mouse development**. *Nature genetics* 2003, **35**(3):215-217.
36. Asli NS, Pitulescu ME, Kessel M: **MicroRNAs in organogenesis and disease**. *Curr Mol Med* 2008, **8**(8):698-710.
37. Carrington JC, Ambros V: **Role of microRNAs in plant and animal development**. *Science* 2003, **301**(5631):336-338.
38. Lu Y, Thomson JM, Wong HY, Hammond SM, Hogan BL: **Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells**. *Dev Biol* 2007, **310**(2):442-453.
39. Havelange V, Garzon R: **MicroRNAs: emerging key regulators of hematopoiesis**. *Am J Hematol* 2010, **85**(12):935-942.
40. Huang Y, Shen XJ, Zou Q, Wang SP, Tang SM, Zhang GZ: **Biological functions of microRNAs: a review**. *J Physiol Biochem* 2011, **67**(1):129-139.
41. Small EM, Olson EN: **Pervasive roles of microRNAs in cardiovascular biology**. *Nature* 2011, **469**(7330):336-342.

42. Garofalo M, Condorelli G, Croce CM: **MicroRNAs in diseases and drug response**. *Curr Opin Pharmacol* 2008, **8**(5):661-667.
43. Akkina S, Becker BN: **MicroRNAs in kidney function and disease**. *Transl Res* 2011, **157**(4):236-240.
44. Farazi TA, Spitzer JI, Morozov P, Tuschl T: **miRNAs in human cancer**. *J Pathol* 2011, **223**(2):102-115.
45. Bader AG, Brown D, Winkler M: **The promise of microRNA replacement therapy**. *Cancer research* 2010, **70**(18):7027-7030.
46. van Rooij E: **The art of microRNA research**. *Circulation research* 2011, **108**(2):219-234.
47. Li W, Ruan K: **MicroRNA detection by microarray**. *Anal Bioanal Chem* 2009, **394**(4):1117-1124.
48. Creighton CJ, Reid JG, Gunaratne PH: **Expression profiling of microRNAs by deep sequencing**. *Brief Bioinform* 2009, **10**(5):490-497.
49. Thomas M, Lieberman J, Lal A: **Desperately seeking microRNA targets**. *Nature structural & molecular biology* 2010, **17**(10):1169-1174.
50. Yang DX, Zhou: **Computational methods for the identification of microRNA targets**. *Dove Medical Press* 2010, **2**:29-32.
51. Pispa J, Thesleff I: **Mechanisms of ectodermal organogenesis**. *Developmental biology* 2003, **262**(2):195-205.
52. Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E: **Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs**. *Nature genetics* 2006, **38**(3):356-362.
53. Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, Seykora JT, Hannon GJ, Millar SE: **The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles**. *Current biology : CB* 2006, **16**(10):1041-1049.
54. Silveri L, Tilly G, Vilotte JL, Le Provost F: **MicroRNA involvement in mammary gland development and breast cancer**. *Reprod Nutr Dev* 2006, **46**(5):549-556.
55. Tucker AS: **Salivary gland development**. *Semin Cell Dev Biol* 2007, **18**(2):237-244.
56. Landais S, Landry S, Legault P, Rassart E: **Oncogenic potential of the miR-106-363 cluster and its implication in human T-cell leukemia**. *Cancer Res* 2007, **67**(12):5699-5707.
57. Olive V, Jiang I, He L: **mir-17-92, a cluster of miRNAs in the midst of the cancer network**. *Int J Biochem Cell Biol* 2010, **42**(8):1348-1354.
58. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ *et al*: **A microRNA polycistron as a potential human oncogene**. *Nature* 2005, **435**(7043):828-833.
59. Mendell JT: **miRiad roles for the miR-17-92 cluster in development and disease**. *Cell* 2008, **133**(2):217-222.
60. Woods K, Thomson JM, Hammond SM: **Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors**. *The Journal of biological chemistry* 2007, **282**(4):2130-2134.
61. Grillari J, Hackl M, Grillari-Voglauer R: **miR-17-92 cluster: ups and downs in cancer and aging**. *Biogerontology* 2010, **11**(4):501-506.
62. Bonauer A, Dimmeler S: **The microRNA-17 approximately 92 cluster: still a miRacle?** *Cell Cycle* 2009, **8**(23):3866-3873.
63. Bueno MJ, Malumbres M: **MicroRNAs and the cell cycle**. *Biochim Biophys Acta* 2011, **1812**(5):592-601.

64. Liu M, Wang Z, Yang S, Zhang W, He S, Hu C, Zhu H, Quan L, Bai J, Xu N: **TNF-alpha is a novel target of miR-19a**. *International journal of oncology* 2011.
65. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR *et al*: **Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters**. *Cell* 2008, **132**(5):875-886.
66. Petrocca F, Vecchione A, Croce CM: **Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling**. *Cancer research* 2008, **68**(20):8191-8194.
67. Emmrich S, Putzer BM: **Checks and balances: E2F-microRNA crosstalk in cancer control**. *Cell Cycle* 2010, **9**(13):2555-2567.
68. Li G, Luna C, Qiu J, Epstein DL, Gonzalez P: **Alterations in microRNA expression in stress-induced cellular senescence**. *Mech Ageing Dev* 2009, **130**(11-12):731-741.
69. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T: **Identification of tissue-specific microRNAs from mouse**. *Current biology : CB* 2002, **12**(9):735-739.
70. Yang JS, Phillips MD, Betel D, Mu P, Ventura A, Siepel AC, Chen KC, Lai EC: **Widespread regulatory activity of vertebrate microRNA* species**. *RNA* 2011, **17**(2):312-326.
71. Benes V, Castoldi M: **Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available**. *Methods* 2010, **50**(4):244-249.
72. Yin JQ, Zhao RC, Morris KV: **Profiling microRNA expression with microarrays**. *Trends Biotechnol* 2008, **26**(2):70-76.
73. Mraz M, Malinova K, Mayer J, Pospisilova S: **MicroRNA isolation and stability in stored RNA samples**. *Biochem Bioph Res Co* 2009, **390**(1):1-4.
74. Sarver AL: **Toward understanding the informatics and statistical aspects of micro-RNA profiling**. *Journal of cardiovascular translational research* 2010, **3**(3):204-211.
75. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR *et al*: **Real-time quantification of microRNAs by stem-loop RT-PCR**. *Nucleic acids research* 2005, **33**(20):e179.
76. Peltier HJ, Latham GJ: **Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues**. *Rna-a Publication of the Rna Society* 2008, **14**(5):844-852.
77. Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N: **Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer**. *BMC molecular biology* 2008, **9**:76.
78. Vecchione A, Croce CM: **Apoptomirs: small molecules have gained the license to kill**. *Endocr Relat Cancer* 2010, **17**(1):F37-50.
79. Shi Y, Jin Y: **MicroRNA in cell differentiation and development**. *Science in China Series C, Life sciences / Chinese Academy of Sciences* 2009, **52**(3):205-211.
80. Enciu AM, Popescu BO, Gheorghisan-Galateanu A: **MicroRNAs in brain development and degeneration**. *Mol Biol Rep* 2011.
81. Thesleff I, Vaahtokari A, Partanen AM: **Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs**. *Int J Dev Biol* 1995, **39**(1):35-50.

82. Simion A, Laudadio I, Prevot PP, Raynaud P, Lemaigre FP, Jacquemin P: **MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2**. *Biochem Bioph Res Co* 2010, **391**(1):293-298.
83. Barshack I, Meiri E, Rosenwald S, Lebanony D, Bronfeld M, Aviel-Ronen S, Rosenblatt K, Polak-Charcon S, Leizerman I, Ezagouri M *et al*: **Differential diagnosis of hepatocellular carcinoma from metastatic tumors in the liver using microRNA expression**. *The international journal of biochemistry & cell biology* 2010, **42**(8):1355-1362.
84. Michon F, Tummers M, Kyyronen M, Frilander MJ, Thesleff I: **Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs**. *Developmental biology* 2010, **340**(2):355-368.
85. Gregory PA, Bracken CP, Bert AG, Goodall GJ: **MicroRNAs as regulators of epithelial-mesenchymal transition**. *Cell Cycle* 2008, **7**(20):3112-3118.
86. Mongroo PS, Rustgi AK: **The role of the miR-200 family in epithelial-mesenchymal transition**. *Cancer biology & therapy* 2010, **10**(3):219-222.
87. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME: **The role of let-7 in cell differentiation and cancer**. *Endocr Relat Cancer* 2010, **17**(1):F19-36.
88. Sonkoly E, Wei T, Janson PC, Saaf A, Lundeberg L, Tengvall-Linder M, Norstedt G, Alenius H, Homey B, Scheynius A *et al*: **MicroRNAs: novel regulators involved in the pathogenesis of psoriasis?** *PLoS One* 2007, **2**(7):e610.
89. Sehic A, Risnes S, Khuu C, Khan QE, Osmundsen H: **Effects of in vivo transfection with anti-miR-214 on gene expression in murine molar tooth germ**. *Physiological genomics* 2011, **43**(9):488-498.
90. Hu JC, Simmer JP: **Developmental biology and genetics of dental malformations**. *Orthod Craniofac Res* 2007, **10**(2):45-52.
91. Sehic A, Risnes S, Khan QE, Khuu C, Osmundsen H: **Gene expression and dental enamel structure in developing mouse incisor**. *European journal of oral sciences* 2010, **118**(2):118-130.
92. Miard S, Peterkova R, Vonesch JL, Peterka M, Ruch JV, Lesot H: **Alterations in the incisor development in the Tabby mouse**. *Int J Dev Biol* 1999, **43**(6):517-529.
93. Cao H, Wang J, Li X, Florez S, Huang Z, Venugopalan SR, Elangovan S, Skobe Z, Margolis HC, Martin JF *et al*: **MicroRNAs play a critical role in tooth development**. *J Dent Res* 2010, **89**(8):779-784.
94. Hayashi T, Koyama N, Azuma Y, Kashimata M: **Mesenchymal miR-21 regulates branching morphogenesis in murine submandibular gland in vitro**. *Developmental biology* 2011, **352**(2):299-307.
95. Nelson PT, Wilfred BR: **In situ hybridization is a necessary experimental complement to microRNA (miRNA) expression profiling in the human brain**. *Neuroscience letters* 2009, **466**(2):69-72.
96. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: **Identification of novel genes coding for small expressed RNAs**. *Science* 2001, **294**(5543):853-858.
97. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G: **miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs**. *Genes & development* 2002, **16**(6):720-728.
98. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T: **A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation**. *Cancer research* 2005, **65**(21):9628-9632.
99. Carraro G, El-Hashash A, Guidolin D, Tiozzo C, Turcatel G, Young BM, De Langhe SP, Bellusci S, Shi W, Parnigotto PP *et al*: **miR-17 family of microRNAs controls**

FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev Biol* 2009, **333**(2):238-250.

100. Lu Y, Okubo T, Rawlins E, Hogan BL: **Epithelial progenitor cells of the embryonic lung and the role of microRNAs in their proliferation**. *Proc Am Thorac Soc* 2008, **5**(3):300-304.
101. Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S: **Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis**. *Circulation research* 2007, **101**(1):59-68.
102. Doebele C, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, Hofmann WK, Zeiher AM, Dimmeler S: **Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells**. *Blood* 2010, **115**(23):4944-4950.
103. Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH: **Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development**. *Hepatology* 2010, **52**(4):1431-1442.
104. Locke M, Hyland PL, Irwin CR, Mackenzie IC: **Modulation of gingival epithelial phenotypes by interactions with regionally defined populations of fibroblasts**. *Journal of periodontal research* 2008, **43**(3):279-289.
105. Cloonan N, Brown MK, Steptoe AL, Wani S, Chan WL, Forrest AR, Kolle G, Gabrielli B, Grimmond SM: **The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition**. *Genome Biol* 2008, **9**(8):R127.
106. Gao FB: **Context-dependent functions of specific microRNAs in neuronal development**. *Neural Dev* 2010, **5**:25.
107. Guo L, Lu Z: **The fate of miRNA* strand through evolutionary analysis: implication for degradation as merely carrier strand or potential regulatory molecule?** *PLoS One* 2010, **5**(6):e11387.
108. Kim YK, Yu J, Han TS, Park SY, Namkoong B, Kim DH, Hur K, Yoo MW, Lee HJ, Yang HK *et al*: **Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer**. *Nucleic acids research* 2009, **37**(5):1672-1681.
109. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT: **c-Myc-regulated microRNAs modulate E2F1 expression**. *Nature* 2005, **435**(7043):839-843.
110. Guil S, Caceres JF: **The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a**. *Nat Struct Mol Biol* 2007, **14**(7):591-596.
111. Chen X, Jorgenson E, Cheung ST: **New tools for functional genomic analysis**. *Drug Discov Today* 2009, **14**(15-16):754-760.

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